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IMMOBILIZATION OF CATALYST BY THE BIOYSYNTHESIS OF BACTERIAL CELLULOSE IN THE PRESNCE OF LACCASE, HORSERADISH PEROXIDASE AND TITANIUM DIOXIDE.

A THESIS

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
APTES	(3-Aminopropyl)triethoxysilane
$NaIO_4$	Sodium Periodate
BC	Bacterial Cellulose
C1	Carbon 1
C2	Carbon 2
CS	Cellulose synthase
DP	Degree of polymerization
ES	Enzyme Substrate
FT-IR	Fourier transform infrared spectroscopy
GK	Glucokianase
HRP	Horseradish Peroxidase
PGM	Phosphoglucomutase
ROS	Reactive Oxygen Species
UGP	UDP-glucose pyrophosphorylase
UDP-Glucose	Uridine diphosphate glucose
TiO_2	Titanium Dioxide
TiO_2-MMT	TiO_2-montmorillonite
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible

ABSTRACT

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IMMOBILIZATION OF CATALYST BY THE BIOYSYNTHESIS OF BACTERIAL CELLULOSE IN THE PRESNCE OF LACCASE, HORSERADISH PEROXIDASE AND TITANIUM DIOXIDE

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Thesis dated December 2013

Enzyme immobilization is a process by which an enzyme is chemically or physically attached to a carrier to impart better physical and chemical properties than free enzymes would exhibit outside of its natural environment. In addition, enzyme immobilization leads to increased stability and ease of separation from products when applied to organic synthesis or industrial processes.

In this study we attempted to immobilize laccase enzyme on bacterial cellulose by standard methods; aminopropylsilation reaction followed by crosslinking the enzyme with gluteraldehyde and by partial oxidation followed by direct reaction with the enzyme. However, both were unsuccessful.

Laccase enzyme was successfully incorporated in bacterial cellulose by adding it to growth media and *Acteobactor xylinum* and carrying out biosynthesis for two to three days. This process was repeated successfully with horseradish peroxidase and the photocatalyst titanium dioxide. The stability, reusability, and activity of the immobilized biocatalyst and photocatlyst were measured.

CHAPTER 1

INTRODUCTION

1.0 Introduction

The goals of this study were to immobilize enzymes horseradish peroxidase (HRP) and laccase, and TiO_2 on bacterial cellulose and study the properties of the supported enzymes and photocatalysts. Enzyme immobilization is a process by which an enzyme is chemically or physically attached to a carrier to impart better physical and chemical properties than the free enzymes would have outside of its natural environment and improve ease of separation from reaction products.[1,2] A metal oxide is often supported on a carrier to allow ease of separation from products, to modify activity of the catalyst and provide an absorbent for the substrate.

There are three main parameters that can be controlled during enzyme immobilization; the type of enzyme and carrier used, and the method of immobilization. The enzyme should be chosen based on the application for which it will be used, its biochemical properties, and its enzymatic parameters. Carriers are defined by unique chemical and mechanical characteristics that should aid in the attachment of the enzyme and also aid in the functionality of the enzyme for its use in a given reaction.1[3] The standard methods of enzyme immobilization are cross linking, encapsulation/inclusion, and attachment to prefabricated carriers.[1] Cross-linking is when an enzyme is attached to

a support with a dual functional linking agent which chemically bonds to both the enzyme and the support.[1,3] Encapsulation/inclusion is when an enzyme is physically entrapped, but not covalently bonded within a polymer network that allows the substrate and products to pass through but retain the enzyme.[3] In the final method a carrier is modified by the addition of functional groups or metals that anchor the enzyme to the support.[3] We were unsuccessful in our attempts cross link enzymes to bacterial cellulose using conventional means. However, we developed a new method to immobilize horseradish peroxidase (HRP) and laccase and photocatalyst titanium dioxide (TiO_2) to bacterial cellulose and study their use. The results of our work will be described latter in this thesis.

A catalyst is a substance that speeds up the rate of a chemical reaction without being consumed. There are many types of catalyst that range from enzymes, photocatalysts, metal catalysts, homogeneous and heterogenous catalysts. Enzymes are biocatalysts, which are naturally occurring proteins that sustain life and catalyze biochemical reactions in cells and can be used for various applications ranging from water purification to organic synthesis.[2,3] Enzymes are found in biological systems which require specific environmental conditions, such as, temperature and pH to function properly. Once an enzyme is removed from its natural biological environment, the enzyme can become unusable and denature. When immobilizing enzymes one has to be aware of the environment needed for the enzyme to function properly. Another characteristic of some enzymes is the need for cofactors.[4] Cofactors are non-protein compounds that are bound to the enzyme in order for it to function.[4] Enzymes have a specific active sites that bind to a substrate to produce a product. Enzymes differ in the mechanism by which they

catalyze reactions. However in general the following steps occur; the substrate binds with the enzyme active site forming an enzyme-substrate complex. The substrate is transformed into one or more products, which are then released from the active site, and the cycle is repeated. This is shown schematically in the equation below.[5]

Enzyme + Substrate → E-S complex → Product and Enzyme

Figure 1. Schematic of enzyme substrate reaction

1.1Background

1.1.1 Immobilization of Enzymes

Enzyme immobilization is a process by which an enzyme is chemically or physically attached to a carrier to impart better physical and chemical properties that free enzymes will have outside of its natural environment and give a longer life span and ease of separation from product.[1,2] This was first demonstrated in beginning of the 19th century with the immobilization of microorganisms to be used industrially for the microbial production of vinegar and the development of the trickling filter or percolating process for waste water clarification.[6] In the 1960s, single enzyme immobilization was developed and used for the isomerization of glucose, and from this advancement; more complex enzyme systems were later immobilized.[6] Industrialization of the use of immobilized enzymes was first reported in 1967 by Chibata and coworkers who developed the immobilization of aminoacylase for the resolution of synthetic racemic D-L amino acids, and lead the way for further industrial process.[6] An increase usage of immobilized enzymes in industry and scientific research led to the development of criteria needed to maximize the efficiency of immobilized enzymes for their use.

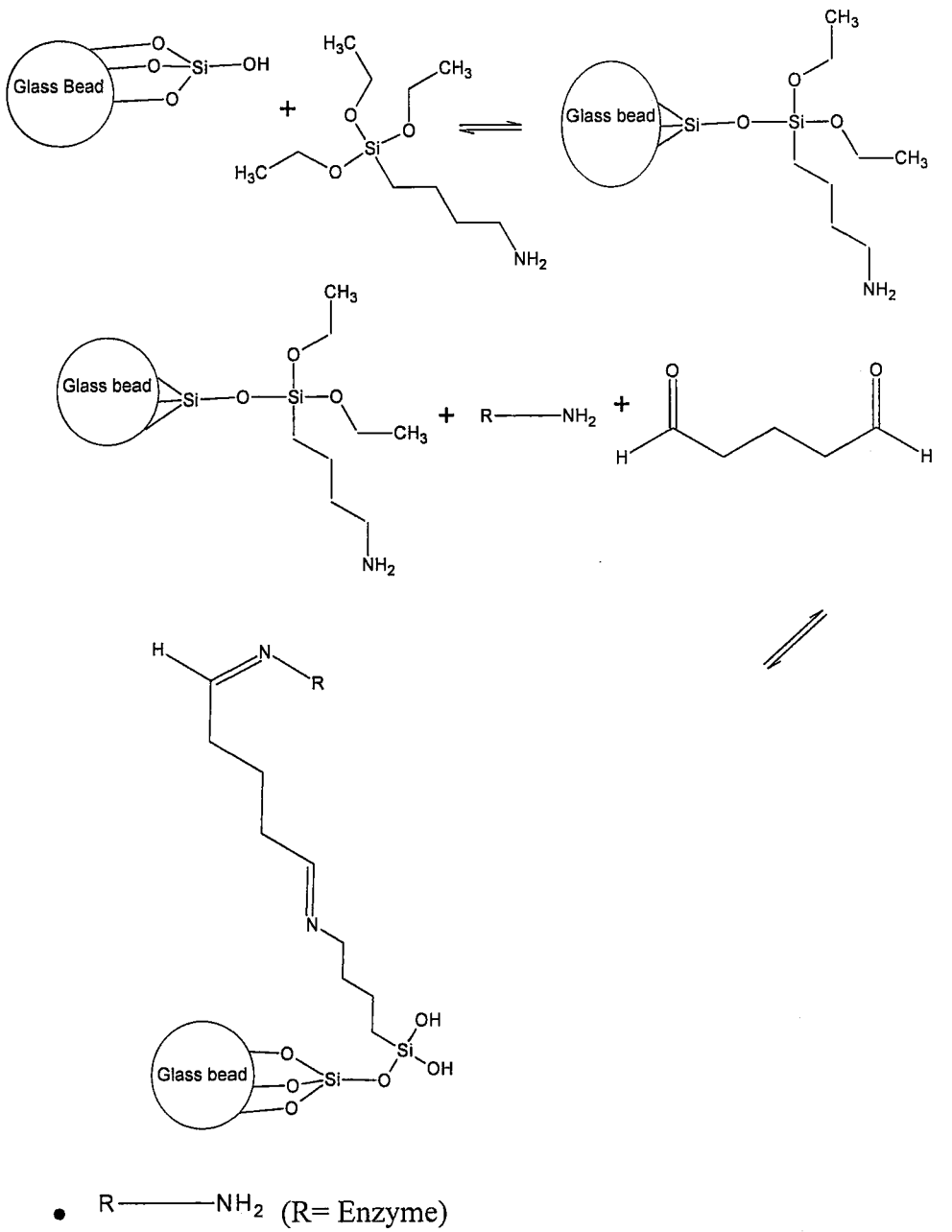
1.1.1.2 Parameters and Standard Methods of Enzyme Immobilization

Parameters must be defined to establish what immobilized system will work best for any application. These parameters include the carrier being used, the enzyme and the immobilization method. The carrier has two main parameters that must be characterized before it is determined if it will be used as a support material for immobilization. These parameters are described by chemical and mechanical properties. Chemical properties include; functional groups, swelling behavior, accessible volume of matrix, pore size, and chemical stability of the carrier. The mechanical properties include; mean wet particle diameter, single particle compression behavior, flow resistance (for fixed bed application), sedimentation velocity (for fluidized bed), and abrasion (for stirred tanks).[7,8,9]

Parameters for enzymatic immobilization include biochemical properties and enzyme kinetic parameters. Biochemical properties include molecular mass, prosthetic groups, functional groups on protein surface, purity (inactivating/protective function of impurities). The enzyme kinetic parameters include specific activity, temperature profiles, kinetic parameters for activity and inhibition, enzyme stability against pH, temperature, solvents, contaminants, and impurities.[10,11,12]

The immobilization methods of enzymes are performed by the standard methods. These methods included crosslinking, attachment to prefabricated carriers, and encapsulation/inclusion.[5] Crosslinking can be performed by direct cross linking via glutaraldehyde, cross linking enzymes and crystallization, which yields cross linked enzyme crystals, and cross linking of physically aggregated enzyme which yields cross

linked enzyme aggregates.[10] Cross linking via glutaraldehyde only works if the carrier has amine groups present on the surface to react with one of the aldehyde groups on the glutaraldehyde structure, while the other aldehyde reacts with the amine group on the enzyme; this is shown in Figure 2. A study by John Wayne used glutaraldehyde to cross link amino acids with polymers for their possible use for molecular weight markers.[11] If amine groups are not present, modification of the carrier must be achieved first, then the cross linking mechanism using glutaraldehyde can be performed.[12] The most common modifier used to add an amine group to a carrier is 3 aminopropyltriethoxysilane (APTES). This method is the most common means of cross linking and is used repeatedly in other studies and is shown in Figure 2.[13,14,15,16]



- $R{-}\!\!-\!\!-NH_2$ (R= Enzyme)

Figure 2. Silanisation of a glass bead by APTES followed by crosslinking of an enzyme via gluteraldehyde.

Attachment to prefabricated carriers are carried out by the functionalization of the support material and then directly attaching the enzyme by ionic, covalent, metal, adsorptive, or bio affinity bonding.[5] Bioaffinity based immobilizations are usually

reversible enabling the reuse of the support matrix and offer the possibility of enzyme immobilization directly from partially purified enzyme preparations or even cell lysates. Bioaffinity is used for a number of interactions including biomolecules and ligands for the immobilization of enzymes, lectins for glycoenzymes bearing appropriate oligosaccharides, cellulose-cellulose binding domain bearing enzymes, and immobilized metal ion surface histidine bearing enzymes.[17] Finally, encapsulation/inclusion can be performed by entrapment in miscelles or membranes. Urease is usually entrapped in a reverse miscelles via hexane and SDS (sodium dodecyl sulfate) reaction, and used for urea detection in blood which signifies kidney malfunction.[18]

1.1.2 Bacterial Cellulose

Bacterial cellulose (BC) is synthesized by bacteria belonging to the genera *Acetobacter, Rhizobium, Agrobacterium*, and *Sarcina*. Its most efficient producers are gram-negative, acetic acid bacteria *Acetobacter xylinum* which has been reclassified as *Gluconacetobacter xylinus*.6 This organism has been designated the model microorganism for basic and applied studies on bacterial cellulose.[19] The reason why the bacteria generates cellulose is unclear, but it has been suggested that it is necessary for their survival, such as to guard against ultraviolet light, or to act as a barrier against to fungi, yeasts and other harmful organisms.[20] Investigators have determined the pathway in which this organism produces cellulose. Biosynthesis of bacterial cellulose by *Actetobactor xylinum* is achieved from a multimeric enzyme complex found in the cellular membrane utilizing glucose through a multistep enzymatic pathway to give the final product of cellulose.[21] Figure 3 shows the enzymatic pathway of the production of cellulose. This understanding along with the manipulation of growth conditions has

enhanced the productivity of cellulose. BC key characteristics are its macromolecular structure, including molecular weight and degree of crystallinity and purity which differs from plant cellulose (PC) by not containing impurities like hemicellulose and lignin.[9,10,11,12, 22]

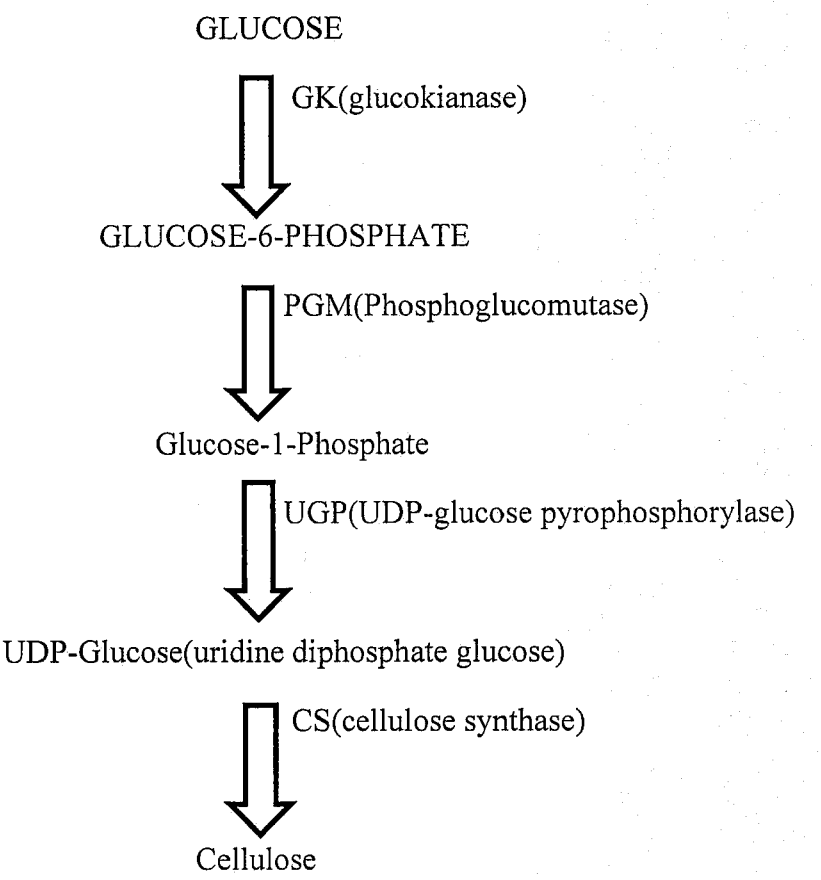


Figure 3. Enzymatic Pathway of Cellulose by bacterial cellulose.

Cellulose is a carbohydrate polymer made up of repeating β 1,4 glucopyranose units and consists of three hydroxyl groups per anhydroglucose unit giving the cellulose molecule a high degree of functionality.[23] There have been numerous studies done on the differences between BC and PC.[9] Yamamota *et al.* were one of the first research groups

to describe the construction of BC, showing that both types of cellulose are chemically identical; however, macromolecular structure and properties of BC differ from PC.[24] Along with not having chemical debris found in PC, BC is unique because it has a hierarchy of order within the structure of cellulose fibers that are produced by the organism.[10,11] This hierarchy begins with chains of BC that aggregate to form 1.5 nm thick subfibrils, and are among the thinnest naturally occurring fibers, comparable to the subelemental fibers of cellulose detected in the cambium of some plants in quinee mucous.[10] BC subfibrils crystallize into microfibrils, these into bundles, and then into ribbons.[6] BC is also distinguished from PC by a high crystallinity index (above 60%) and a high degree of polymerization (DP), usually between 2000 and 6000 but in some cases as high as 16,000 to 20,000 whereas the average DP of plant cellulose varies from 13,000 to 14,000.[25] These properties make BC a desirable material for a wide range of applications, including artificial skin for wound covering, scaffold for tissues engineering and blood vessels, pervaporation of aqueous organic mixtures, and immobilization of catalyst.[4,26,27,28]

Bacterial cellulose has been used as a catalyst support by crosslinking, encapsulation, and modification of a prefabricated carrier. Barbara R. Evansa *et al.* used bacterial cellulose as a prefabricated carrier by modifying with palladium to generate hydrogen when incubated with sodium dithionite. This study showed that cellulose had advantages over the common used support material, polyelectrolyte membrane including a higher thermal stability and lower gas cross over.

Glucoamylase, an enzyme that hydrolyzes starch, has been immobilized on bacterial cellulose via cross linking by the reaction of cellulose with an epoxy group and

then tethered via reaction with gluteraldehyde.[29] This method enhanced the stability of the enzyme; allowing it to function over a wider range of pH and temperature than the free enzyme.[29] An adsorption cross linking method was also used to immobilize laccase and glucose oxidase on cellulose, produced by *Acetorbactor xylinum* in coconut milk, to evaluate the effects of different membrane sizes, surface areas, adsorption times and the amount of enzyme immobilized on the bacterial cellulose. The highest activity of the immobilized laccase varied with attachment method, and was found to be pH 3.5 and 4.0 for the adsorption and adsorption-crosslinking method respectively at 60 °C. For the glucose oxidase the optimum pH also varied by immobilization method and was found to be at pH 5.0 and 6.0 for immobilization by adsorption and adsorption-crosslink, at 35 °C.[28]

1.3 Laccase

Laccase (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductase), which is an oxidoreductase enzyme that was first believed to only occur as the plant ascorbate oxidase and the mammalian plasma protein ceruloplasmin, was later discovered in many white rot fungi (*Ascomycetes, Deuteromycetes* and *Basidiomycetes*), which are involved in lignin metabolism.[14,30,31]

Laccase is characterized by having an active site that contains 4 catalytic copper atoms. These copper atoms are located in sites described as T1 and T2/T3. One copper is located at the T1 site, where the reducing substrate binds; the other three coppers are clustered in the T2/T3 site in which molecular oxygen binds.[32]

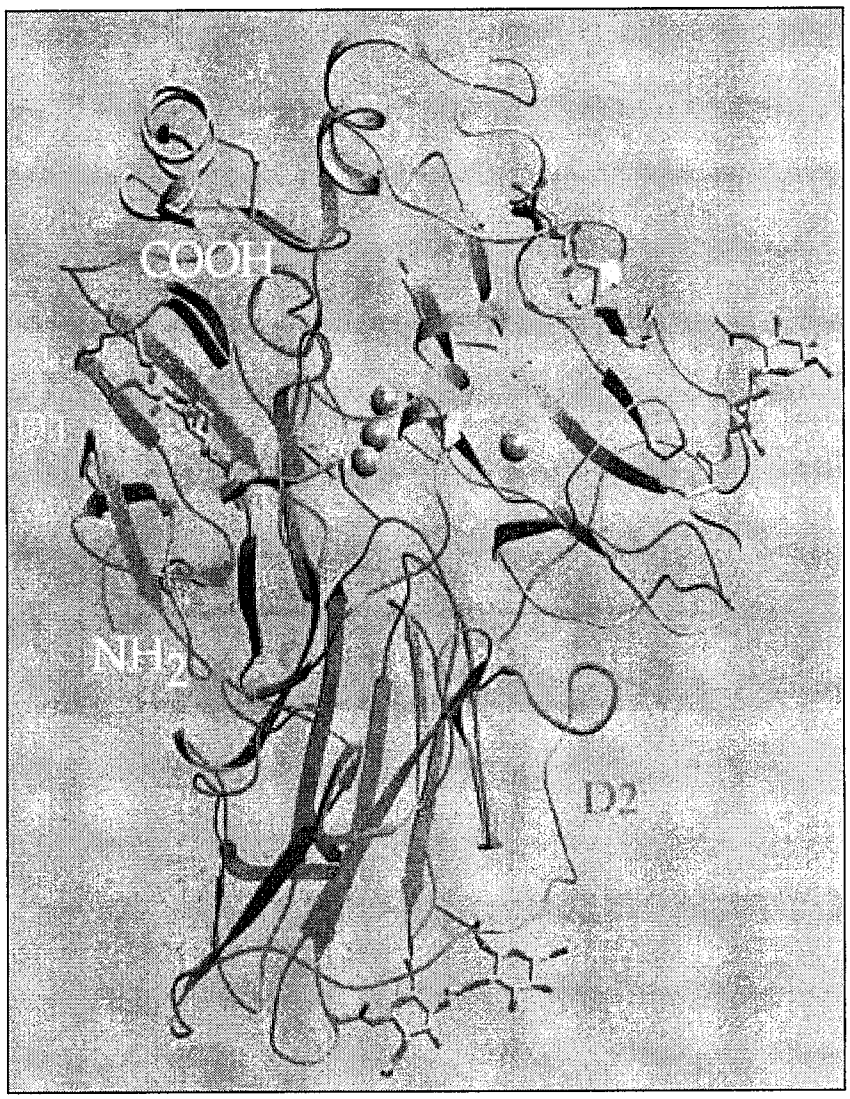


Figure 4. Ribbon diagram of laccase. The arrangement of the domain structure is depicted in different color coding (D1–D3). Copper ions are drawn as blue spheres, carbohydrates and disulfide bonds are included as stick models.[33]

Laccases catalyze the one electron oxidation of phenolic compounds while simultaneously reducing oxygen to water.[34] The reaction mechanism is the ping pong type since the enzyme switches from the stable native reduced state to the stable oxidized state and release water.[35] Oxygen, the electron acceptor, can bind to the enzyme to be reduced to water before the electron donor phenol or 2,2'-azino-bis(3-

ethylbenzthiazoline-6-sulphonic acid (ABTS)) binds to the enzyme to convert to the second product.[16]

The steady-state rate equation for the ping-pong mechanism assuming that the reversed reaction is negligible is:

$$v = \frac{V_{max}}{K_{M,B}[A] + K_{M,A}[B] + [A][B]} \qquad \text{(Eq. 1}$$

Figure 5. Steady state rate equation for the ping-pong mechanism.

During a steady-kinetics experiment with a bisubstrate reaction, one substrate concentration is varied while the other is held constant. For laccase, the oxygen concentration is assumed to be constant (at 8.04 mg/L) for the duration of the enzyme assay if the substrate conversion does not exceed 10 % and if the substrate concentration largely exceeds that of the enzyme. It is then possible to rearrange the bisubstrate equation to the general Michaelis-Menten form with respect for both substrates.[17]

Laccase activity can be extended to non-phenolic compounds allowing this enzyme to be used in a wide range of industrial process and environmental bioremediations. These applications include: removal of environmental pollutants (chlorophenols, dyes, herbicides etc.), effluent decolorization and detoxification for pulp bleaching, removal of phenolics from wines, organic synthesis, biosensors, synthesis of complex medical compounds and dye transfer blocking functions in detergents and washing powders, many of which have been patented.[36]

Laccase immobilization has been extensively studied with a wide range of methods and substrates.[37] Mogharabi *et al.* immobilized laccase by entrapment in an alginate gelatin mixed gel and used this system for the decolorization of dyes.[38] Lu *et al.* reported the immobilization of laccase in alginate chitosan microcapsules for the decolorization of alizarin red using ABTS as a redox mediator.[39] They showed laccase stability was enhanced by immobilization compared to the free enzyme. They also tested the immobilized enzyme for reusability and observed that the immobilized laccase could be reused three times while retaining 35% of its activity. Laccase has also been immobilized on mesopourus molecular sieves by cross linkage with gluteraldehyde and its activity and stability was examined. They used indigo carmine as a substrate and ABTS as a redox mediator to examine the parameters of the immobilized enzyme compared to the free enzyme. The authors found that the immobilized enzyme stability, including thermal, pH and operational stability, were improved after immobilization compared to free enzyme.[40] Laccase has been immobilized on a TiO_2-montmorillonite (TiO_2-MMT) complex and tested for its activity and stability. The complex was prepared by blending TiO_2 sol and MMT followed by calcination at 800 °C in air for 2 h, identified as TiO_2-MMT800, and then laccase was immobilized by adsorption. Figure 5 shows a schamatic of the laccase immobilzed on the TiO_2-MMT. The study of immobilized laccase activity revealed that the optimum pH and temperature were pH 3 and 60 °C. The authors determined from the kinetic studies that laccase immobilized on TiO_2-MMT800 complexes had a good affinity to the substrate.[41]

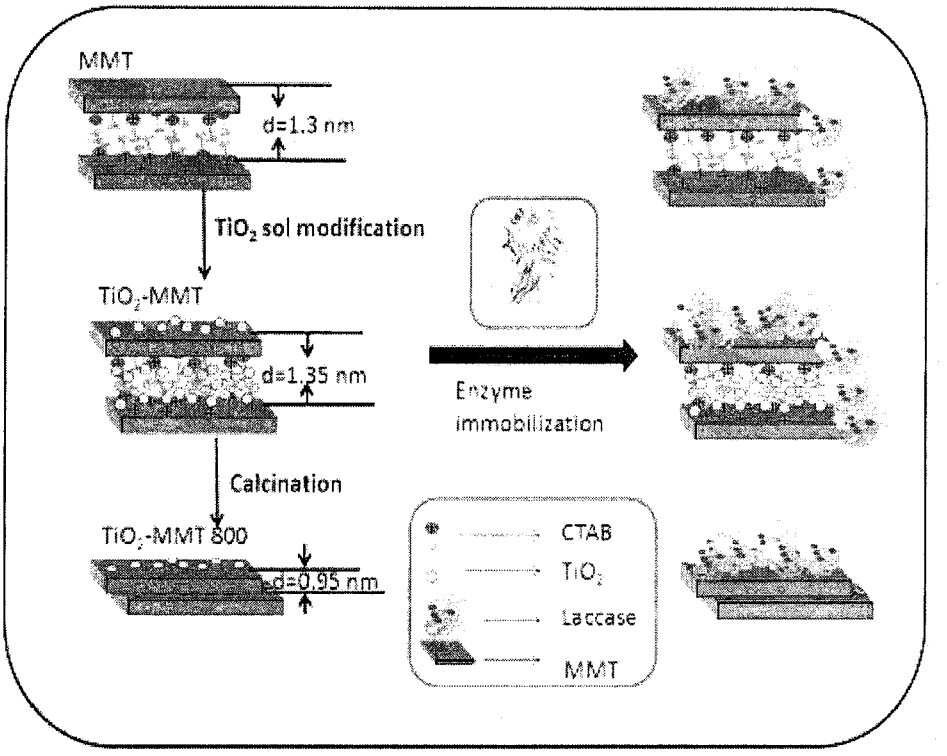


Figure 6. Schematic of immobilization of laccase on TiO_2-MMT.

1.4 Horseradish peroxidase

Horseradish peroxidase (HRP), is found in a perennial herb, horseradish, cultivated for the culinary value of its roots.[21] The root of the plant contains a number of distinctive peroxidase isoenzymes of which the C isoenzyme (HRP C) is the most abundant. This enzyme is a heme- containing enzyme that uses hydrogen peroxide to oxidize a variety of substrates. In 1976, Welidner determined the sequencing of the amino acid sequence consisting of 308 residues.[42] There are nine potential N-glycosylation sites that can be recognized in the primary sequence. Yang *et al.* characterized the branched heptasaccharide, which accounts for 75 to 80% of the

glycans.[28] The carbohydrate profile of HRP C is heterogeneous; however, many minor glycans have also been characterized.[43]

Figure 7. Three-dimensional representation of the X-ray crystal structure of horseradish peroxidase isoenzyme C. The heme group (colored in red) is located between the distal and proximal domains which each contain one calcium atom (shown as blue spheres). α-helical and β-sheet regions of the enzyme are shown in purple and yellow, respectively.[44]

Catalysis by horseradish peroxidase starts with the oxidation of the native enzyme by hydrogen peroxide forming an active intermediate enzymatic form called Compound I. Compound I then accepts an aromatic compound into its active site and carries out a one electron oxidation. A free radical is produced and released into solution leaving the enzyme in the Compound II state. Compound II carries out another one electron oxidation on a second aromatic molecule, and then releases another free radical returning the enzyme to its native state, thereby completing the cycle. The overall reaction is

described by equations 1-4, where E represents the enzyme and A the aromatic compound.[45]

$$E + H_2O_2 \rightarrow E_i + H_2O \quad \text{(Eq. 1)}$$

$$E_i + AH \rightarrow E_{ii} + \bullet AH \quad \text{(Eq. 2)}$$

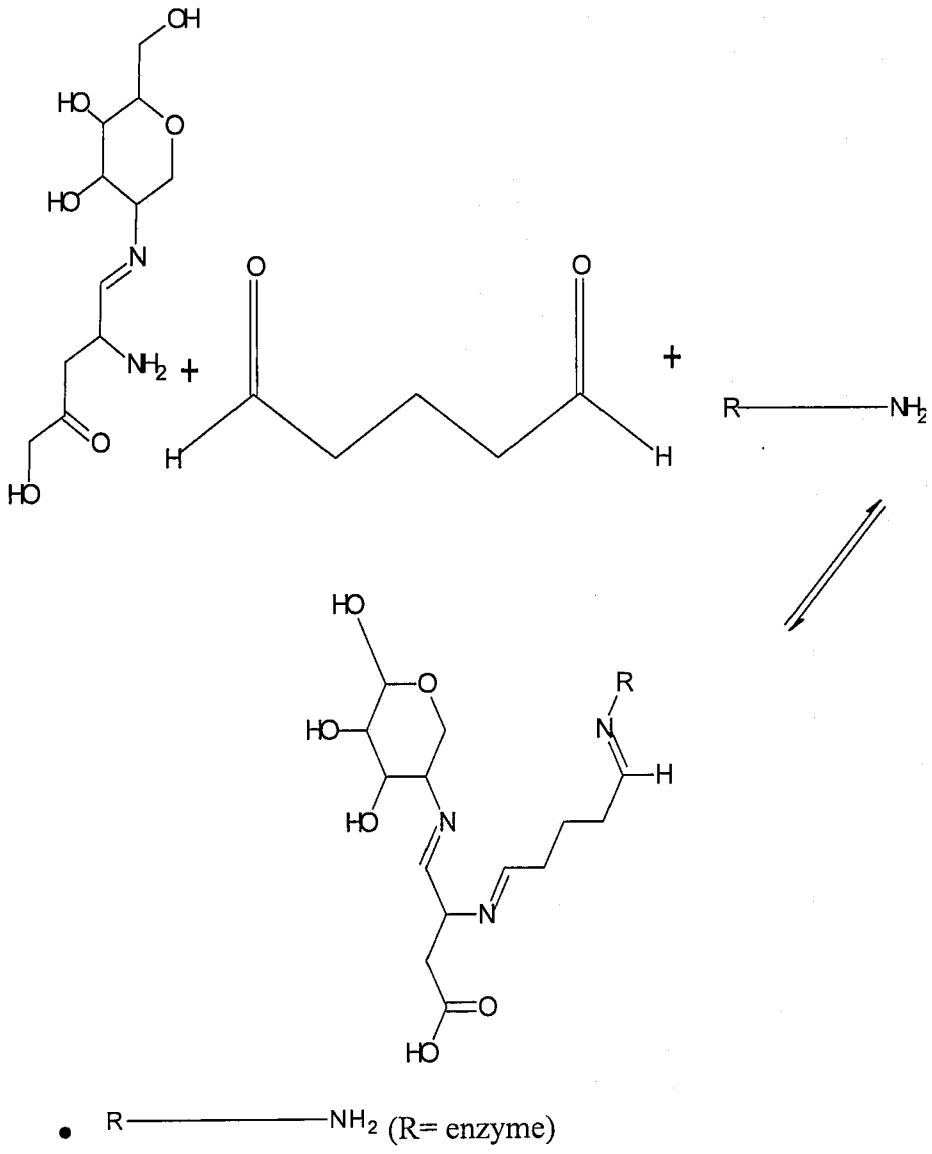
$$E_{ii} + AH_2 \rightarrow E + \bullet AH + H_2O \quad \text{(Eq. 3)}$$

$$H_2O_2 + 2AH_2 \rightarrow 2 \bullet AH + 2H_2O \quad \text{(Eq. 4)}$$

Figure 8. Horseradish peroxidase enzymatic reaction.

Horseradish peroxidase (predominantly HRP C) is used for a variety of applications including organic synthesis biotransformation coupled enzyme assays, chemiluminescent assays, immunoassays and the treatment of waste waters. Horseradish peroxidase has been used in bench scale organic synthesis for N- and O-dealkylation, oxidative coupling, selective hydroxylation and oxygen-transfer reactions.[23,46]

Mohamed *et al.* immobilized horseradish peroxidase on a composite membrane by coating it with nonwoven polyester fabric and chitosan glutamate and then cross linked the enzyme to this carrier using gluteraldehyde. They performed an enzymatic assay on the free and immobilized enzyme by monitoring the oxidation of guaiacol and found that the immobilized enzyme was more stable than the free enzyme.[47] A schematic of the cross linkage reaction is shown in Figure 9.



• R——NH2 (R= enzyme)

Figure 9. Cross linkange reaction between chiostan glutamate and horseradish peroxidase via gluteraldehyde.

In another study by Baccar *et al.*, they reported a prefabricated carrier method by immobilizing horseradish peroxidase on layered double hydroxides (LDH) modified gold surfaces. The hydrotalcite LDH was prepared by co-precipitation in constant pH and in ambient temperature. The immobilization of the peroxidase on layered hybrid materials was realized via electrostatic adsorption auto assembly process. They used this system as a biosensor for hydrogen peroxide.[48] Horseradish peroxidase has been immobilized on

other support systems such as cellulose, magnetite, gold electrodes, O-carboxymethylated chitosan/sol gels, mesoporous silica and modified poly (ethylene terephthalate) grafted acrylamide fiber. In each study, the horseradish peroxidase activity was enhanced compared to the free enzyme.[49,50,51,52,53]

1.5 Titanium Dioxide

Titanium dioxide is a naturally occurring mineral that has three forms; anatase, brookite and rutile.[54] It is utilized as a white pigment in paints, food additives, pharmaceuticals, and cosmetics.[55] TiO_2 is also a semiconductor photocatalysis which by the following steps: When light of a wavelength greater than the band gap irradiates TiO_2; electrons are excited from the valence band to the conduction band leaving a positive hole (h^+). The electrons (e^-) in the conduction band and positive holes (h^+) in the valence band cause reduction and oxidation reactions, respectively, resulting in the formation of reactive oxygen species which can degrade organic pollutants or inactivate microorganisms. Specifically, an O_2 molecule scavenges the e- from the conduction band of the TiO_2 semiconductor forming a superoxide radical (O_2^-). A H_2O molecule or hydroxide ion (OH^-) is oxidized by the h+ to form $OH\bullet$[35] The reactive oxygen species (ROS), O_2^- and OH once formed can react with harmful organic materials and decompose the material to less harmful byproducts.[26]

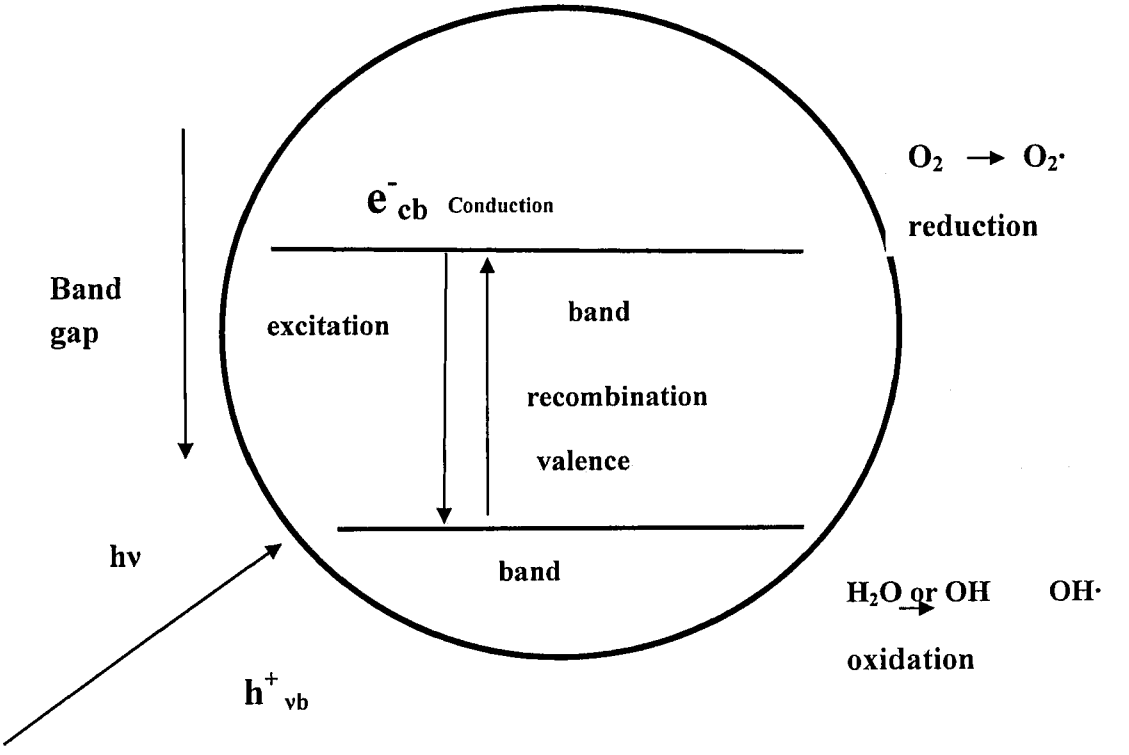


Figure 10. Diagram of semiconductor photocatalysis in water.[56]

Immobilization of TiO_2 has been successfully achieved by using the standard methods as described previously.[28,31] One study showed the immobilization of TiO_2 on a cellulose membrane or as a nanoparticle film by using the crosslinking method via gluteraldehyde, to enhance treatment of a suspension of *Escherichia coli* (*E. coli*). They observed complete inactivation of *E. coli* suspensions after treatment with the immobilized TiO_2 under UV light.[57,58] Hanel *et al.* immobilized TiO_2 on glass beads. They first made a hydrogel with TiO_2 and then attached it to the modified glass beads and later doped with boron.[59]

1.6 Chlorophenols

Chlorophenols are organochloride of phenol that contain one or more covalently bonded chlorine atoms.[60] They are toxic compounds that are present in drinking-water as a result of the chlorination of phenols during disinfection, as by-products of the reaction of hypochlorite with phenolic acids, as biocides, or as degradation products of phenoxy herbicides.[26] They are also used extensively in fungicide, herbicide, wood preservative and pesticide intermediate and because of their carcinogenicity, toxicity and persistence, chlorophenols are listed among top priority control pollutants by the EPA.[61]

The removal of these compounds from waste water has been extensively studied by using enzymes and photocatlayst TiO_2.[62,63,64] The use of horseradish peroxidase for the removal chlorophenols has one major disadvantage, it produces polymers through a process called oxidative coupling. This makes it difficult to remove the polymers formed once oxidation of the chlorophenols are completed.[65] Tatsum *et al.* immobilized HRP on magnetite and used this for the removal of chlorophenols in waste water. They showed that this process does remove some of the polymers formed during the reaction, suggesting that immobilization not only improves the function of the enzyme, but aids in the efficiency of removing cholorophenols and byproducts.[30] Studies show that the photocatalytic oxidation of cholorophenols by TiO_2 is very effective method for their removal from waste water.[66]

CHAPTER 2

MATERIALS AND EXPERIMENTAL

2.0 Materials

All the chemical reagents used in these experiments were obtained from commercial sources as guaranteed grade reagents and used without further purification. Laccase, horseradish peroxidase, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Sigma-Aldrich 30931-67-0), phosphate-citrate buffer tablets pH 5(Sigma-Aldrich P4809), phenol, potassium hydrogen phosphate (potassium phosphate dibasic), potassium dihydrogen phosphate (potassium phosphate monobasic), 4-aminoantipyrine, and 30% hydrogen peroxide, peptone, yeast extract, citric acid, and glucose was purchased from Sigma-Aldrich. *Actetobactor xylinum* (ATCC 700178) was purchased from ATCC and *Candida albicans* (clinical isolate H317) was given by Dr. Logan from Clark Atlanta University. Glass sterile multiwell plates with 6 wells (Catalog number: NC9451103), 8 w UV light (UVP Multira) were purchased from Fisher Scientific. Plastic sterile 15.00 mL and 50.00 mL conical test tubes were purchased from VWR. Degussa P25 (TiO_2) was purchased from Degussa U.S.A. Disodium phosphate and magnesium sulfate were purchased from Fischer Scientific.

2.1 Biosynthesis of Bacterial Cellulose

Schramm and Hestrin's (HS) broth was prepared by adding 20.23 g of glucose, 5.18 g of peptone, 2.70 g of Na_2HPO_4, 5.03 g of yeast extract, 0.53 g of $MgSO_4 \cdot 7H_2O$

and 1.17 g of citric acid in a 1.00 L volumetric flask with 1.00 L of sterile water. An aliquot of 3.00 mL of HS broth was pipetted in a sterilized 15.00 mL conical test tube aseptically. Then 1.00 mL of *Acetobactor xylinum* cells suspension was added aseptically. The suspension was incubated at 27 °C for 3 days, frozen, and used as controls in future experiments.

2.2 Biosynthesis of Bacterial Cellulose in the Presence of Laccase

Samples of 0.00150 g of laccase was weighed and dispensed into 10, 15.00 mL sterilized conical test tubes. Aliquots of 4.00 mL suspensions of HS broth and *Acetobactor xylinum* cells were then added aseptically. The suspensions were incubated at 27 °C for a total of 36 days and individually tested for enzymatic activity periodically. This data is shown in chapter 3.4.

2.3 Buffer solutions

2.3.1 Phosphate buffers

A 87.09 g (.5 mol) of K_2HPO_4 and 68.05 (.5 mol) of KH_2PO_4 was weighed and dispensed into two separate 500.00 mL volumetric flask with water giving a final concentration of 1 M. This stock was used to make buffers pH 5, pH 7 and pH 7.8. They were prepared as follows: An aliquot of 8.5 mL of K_2HPO_4 solution was added to 91.5 mL of KH_2PO_4 and diluted with distilled water in a 1.00 L volumetric flask to give a final pH of 5. A 57.89 mL aliquot of K_2HPO_4 and a 98.49 mL aliquot of KH_2PO_4 was added to a 500.00 mL volumetric flask with water (0.2 M) to give a final pH of 7. A 90.8 mL aliquot of K_2HPO_4 and 9.2 mL of KH_2PO_4 was added to a 500.00 mL volumetric flask

containing water (0.1 M) to give a final pH of 7. 8. The pH was adjusted with HCL using an Accumet Research pH meter (Fisher Scientific).

2.3.2 Citrate-Phosphate Buffer pH 5

A phosphate-citrate tablet (Sigma-Aldrich P4809) was dissolved in 100.0 mL of distilled water in a volumetric flask giving a final pH 5.

2.4 ABTS Solution

A (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) ABTS tablet (Sigma-Aldrich 30931-67-0) was added and then stirred in a 100.0 mL volumetric flask containing phosphate-citrate buffer pH 5 as mentioned in section 2.3.2, giving a final concentration of 0.05 M. The solution was covered with parafilm and kept on ice until use. The pH was verified by using an Accumet Research pH meter (Fisher Scientific).

2.5 Suspension of Free Laccase

Samples of 0.00150 g of laccase was weighed and dispensed in 10, 15.00 mL sterilized conical test tube containing 4.00 mL 0.1 M phosphate buffer (pH 5) which is described in section 2.3.1. These samples were incubated at 27 °C up to 36 days. These results are shown in chapter 3.1 and 3.7.

2.5.1 Enzymatic Assay of Free Laccase

A 1.5 mL suspension of free enzyme was pipetted into a sterile conical test tube that contained 1.5 mL chilled ABTS solution, then quickly poured into a cuvette and the optical density was read by a Beckman coulter 800 spectrophotometer at 415 nm. The

spectrophotometer was set at kinetic scan for 5 min with a sample reading taken every 60 s. This data is discussed in chapter 3 section 1.

2.6 Enzymatic Assay of Immobilized Laccase

Biosynthesis of bacterial cellulose in the presence of laccase was carried out for 3 days as described in section 2.2. The cellulose grown in the presence of laccase was washed 3 times with phosphate buffer pH 5, and added sterile conical test tube containing 1.5 mL of chilled ABTS and reacted for 1 min. The solution was poured off the cellulose pellicle into a plastic cuvette and the optical density was read by a Beckman coulter 800 spectrophotometer at 415 nm. The solution was then poured back in the test tube containing the immobilized laccase on cellulose and reacted for another min. This procedure was repeated for 5 min. This data is discussed in chapter 3.7.

2.6.1 Reusability and Storage of Immobilized Laccase Enzyme

Biosynthesis of cellulose was carried out in the presence of laccase for 3 days as described in section 2.2. The activity of immobilized laccase was measured by the enzymatic assay described in section 2.5.2. The sample was then washed with phosphate buffer pH 5 and stored in a 15.00 mL conical flask containing 10.00 mL of phosphate buffer pH 5 at 8 °C for 30 days. Periodically, the immobilized laccase activity was measured by the enzymatic assay.

2.6.2 Reusability of Immobilized Laccase Enzyme in Continuous Reaction

Biosynthesis of cellulose was carried out in the presence of laccase for 3 days as described in section 2.2. The activity of the immobilized laccase was measured by the

enzymatic assay as described in section 2.5.2. This was repeated 11 times with washing in between each assay. This data is show in chapter 3.9.

2.7 Essay Solutions for Horesradish Peroxidase

2.7.1 Phenol/4 Aminoantipyrine solution

A sample of 0.810 g of (0.17 M) phenol and a sample of 0.025 g of (0.0025 M) 4-aminoantipyrine was added (in the dark) to a 50.00 mL volumetric flask containing phosphate buffer pH 7 as described in section 2.3.1. This solution was stirred and stored at room temperature until further use.

2.7.2 Hydrogen Peroxide

A 0.0017 M solution of hydrogen peroxide was made by diluting 1.0 mL of 30% H_2O_2 in 100.00 mL of deionized water in a volumetric flask. A 1.00 mL aliquot was then pipetted in 50.00 mL volumetric flask containing phosphate buffer pH 7.

2.8 Suspension of Free Horseradish Peroxidase:

Samples of 10.01 mg of horseradish peroxidase (HRP) was weighed and dispensed in 8, 15.00 mL sterilized conical test tube containing 4.0 mL of 0.2 M phosphate buffer pH 7. The test tubes were incubated at 27 °C for 8 days and tested for enzymatic activity each day. The pH was verified by using an Accumet Research pH meter (Fisher Scientific).

2.8.1 Enzymatic Assay of Free Horseradish Peroxidase

An aliquot of 1.5 mL of free horseradish peroxidase solution was pipetted into a test tube containing 1.5 mL 0.17M/0.0025 M solution of phenol and 4-aminoantipyrine and 1.5 mL of hydrogen peroxide then quickly poured into a cuvette. The optical density was read by a Beckman coulter 800 spectrophotometer at 510 nm. The spectrophotometer was set at kinetic scan for 5 min with a sample reading taken every 60 s.

2.9 Biosynthesis of Cellulose in the Presence of Horseradish Peroxidase

Samples of 10.01 mg of horseradish peroxidase (HRP) was weighed and dispensed in 8, 15.00 mL sterilized conical test tube containing 4.0 mL suspensions of HS broth and *Acetobactor xylinum* cells. The suspensions were incubated at 27 °C for 8 days and tested for enzymatic activity each day. This data is shown in chapter 3 section 7.

2.9.1 Enzymatic Assay for Immobilized Horseradish Peroxidase

Samples of immobilized horseradish peroxidase on cellulose were synthesized for 4 days as described in section 2.6. These samples were washed 3 times with phosphate buffer pH 7, and the washed immobilized horseradish peroxidase on cellulose was added to a test tube which contained 1.5 mL of phenol/4-aminoantipyrine solution and 1.5 mL hydrogen peroxide and reacted for 1 min. After 1 min, the solution was poured off the immobilized horseradish peroxidase into a cuvette and the optical density was read by a Beckman coulter 800 spectrophotometer at 510 nm. The solution was then poured back

in the test tube containing the immobilized laccase and reacted for another min. This procedure was repeated for 5 min. The results are discussed in chapter 3.7.

2.9.2 Reusability and Storage of Immobilized Horseradish Peroxidase

Biosynthesis of cellulose was carried out in the presence of horseradish peroxidase for 4 days as described in section 2.4. The activity of immobilized horseradish peroxidase was measured by the enzymatic assay described in section 2.9.1. The sample was then washed with phosphate buffer pH 7 and stored in a 15.0 mL conical flask containing 10.0 mL of phosphate buffer pH 7 at 8 °C for 21 days. Periodically, the immobilized horseradish peroxidase activity was measured. Results are discussed in chapter 3.9.

2.9.3 Reusability of Immobilized Horseradish Peroxidase in Continuous Reaction

Biosynthesis of cellulose was carried out in the presence of horseradish peroxidase for 4 days as described in section 2.6. The activity of the immobilized horseradish peroxidase was measured by the enzymatic assay described in section 2.8. This was repeated 6 times consecutively, with washing in between each assay.

2.10 Degradation of 2 Chlorophenol by Immobilized Horseradish Peroxidase Colorimetric Assay

Samples of 2 chlorophenol were prepared as described in section by diluting 40 μL (3.95×10^{-4} mol) of 2-chlorophenol in 50 mL of pH 7.8 phosphate buffer to a concentration of 7.9×10^{-3} M. An aliquot of 5.00 mL of solution was then diluted in a 50 mL volumetric flask containing phosphate buffer pH 7.8 and 0.025 g of

4-aminoantipyrine making a final concentration of 7.9 X 10^{-4} M. Three 2 mL aliquots were pipetted into 3 test tubes containing 2.00 mL hydrogen peroxide (0.0017M) pH 7.8 and 5.00 mL phosphate buffer pH 7.8 making a final concentration of 7.9 X 10^{-5} M. Immobilized horseradish peroxidase on cellulose and regular bacterial cellulose (no horseradish peroxidase) is first washed three times in distilled water then added to each test tube for 1 min. The solution is poured into a cuvette and the optical density is read at 510 nm. The solution is then poured back in the test tube and this procedure is repeated for 10 min. Results are discussed in chapter 3.11.

2.10.1 Degradation of 2 Chlorophenol by Free Horseradish Peroxidase Colorimetric Assay

Samples of 2 chlorophen ol were prepared as described in section 2.9. 10.01 mg of horseradish peroxidase was added to test tubes containing 2.00 mL hydrogen peroxide and 5.0 mL of phosphate buffer pH 7.8. This solution was then quickly poured into a cuvette and the optical density was read by a Beckman coulter 800 spectrophotometer at 510 nm. The spectrophotometer was set at kinetic scan for 10 min with a sample reading taken every 60 s.

2.11 FT-IR Spectroscopy of Immobilized Horseradish Peroxidase

Immobilized horseradish peroxidase on cellulose was dried in a vacuum oven at 60 °C for 4 h. The sample was then analyzed using an ATR-FTIR at 4 resolution and 24 scans. Free horseradish peroxidase and regular cellulose (no horseradish peroxidase) was also analyzed using the same parameters. Results are discussed in chapter 3.12.

2.12 Biosynthesis of Cellulose in the Presence of Titanium Dioxide (TiO_2)

Samples of 3.0 mg of TiO_2 was weighed (in the dark) and dispensed in 7, 15.00 mL sterilized conical test tubes. A 4.0 mL suspension of HS broth and *Acetobactor xylinum* cells were then added to the test tubes aseptically. The suspensions were incubated and shaken at 27 °C for 7 days in the dark and tested for photocatalytic activity each day for 7 days as described in section 2.12.

2.12.1 Catalytic Assay for Immobilized and Free TiO_2

Cells of *Candida albicans* were cultured first in yeast extract peptone dextrose broth (YEPD) for 24 h and then samples of the colonies were transferred aseptically in 30.00 mL of YEPD broth for 6 h at 37 °C. The cells were then vortex three times and two 30.00 μL aliquots were pipetted in a haemocytometer. The cells were counted using a light microscope of 100X magnification and the concentration was determined and a stock was made at 10^5 colony forming units (CFU). A 6 well microtitier plate contained 4 mL of stock *Candida albicans* suspension, 0.5 mL of bicarbonate solution pH 8.1 and immobilized TiO_2. There were two controls for this experiment. The first control contained a 4.5 mL stock suspension of *Candida albicans* cells with 0.5 mL of bicarbonate solution pH 8.1. The second control contained 3 mg free TiO_2 with 4 mL stock *Candida albicans* cell suspension and 0.5 mL of bicarbonate solution pH 8.1. The microtiter plate was placed under UV light for 24 h. Samples of 100 μL aliquots of each sample were pipetted and evenly spread on YEPD agar plates and incubated at 37 °C for 24 to 48 h and counted for the survival of *Candida albicans*. This experiment was also performed in the dark as a control. Results are discussed in chapter 3.8.

2.12.2 Degradation of 2-Chlorophenol by Immobolizd TiO_2

Samples of 2 chlorophenol were prepared by diluting 40 μL (3.95×10^{-4} mol) of 2 chlorophenol in 50.00 mL of pH 7.8 phosphate buffer to a concentration of 7.9×10^{-3} M. An aliquot of 5.00 mL of solution was then diluted in a 50.00 mL volumetric flask containing phosphate buffer pH 7. Aliquots of 10.00 mL samples were pipetted to a 6 well microtiter plate which contained 3 mg of free TiO_2 in one well, 3 mg immobilized TiO_2 in another well and bacterial cellulose (no TiO_2) in the final well. This was then wrapped in aluminum foil and shaken in the dark for 1h. The plate was then exposed to UV light for 6 h and a 200 microliter sample was drawn every 30-60 min, diluted to 2.0 mL (7.9×10^{-5} M), centrifuged at 4000 rpm for 3 min at 25 °C, and then the optical density was read at 275 nm. Results are discussed in chapter 3.11.

2.13 Aminopropylsilation of Bacterial Cellulose

A 0.255 g sample of cellulose was placed in a schlenk tube fitted with an o-ring joint and a Teflon stopcock. The tube was flushed with nitrogen and evacuated three times before 25.50 mL of toluene and 1.00 mL of ethanol was added. Under nitrogen, 0.5 mL of (3-Aminopropyl)triethoxysilane (APTES) was added into the reaction tube while the solution was stirred. The reaction was placed in an oil bath and heated to 70 ° C 24 h. The cellulose was washed with toluene (2X), water (2X) and ethanol (2X) before it was placed in an oven and dried at 110 °C for 30 min. The sample was then analyzed using an ATR-FTIR at 4 resolution and 24 scans.

2.14 Oxidation of Bacterial Cellulose by Sodium Meta-Periodate Reaction

A sample of 2.61 g of (0.122 M) $NaIO_4$ was dispensed (in the dark) in a 250 mL beaker which contained 2.03 g of bacterial cellulose and 100 mL of water, covered in aluminum. This was shaken for 24 h then dried at 60 C for 4 h. The sample was then analyzed using an ATR-FTIR at 4 resolution and 24 scans.

CHAPTER 3

RESULTS AND DISCUSSION

3.0 Introduction

Immobilization of enzymes on supports has allowed them to be utilized for chemical reactions and industrial applications.[6] We were unsuccessful immobilizing laccase and horseradish peroxidase enzymes, and TiO_2 with bacterial cellulose using conventional methods. Therefore, we have developed an alternative method for the immobilization of laccase and horseradish peroxidase enzymes, and TiO_2 with bacterial cellulose and characterized their catalytic activity with selective substrates as described below.

3.1 Activity of laccase for Oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

The laccase enzyme was purchased as a crude extract which we found to have a water soluble and insoluble fraction. The crude extract, the soluble, and insoluble fraction all exhibited activity for the catalytic oxidation of ABTS. For example, a 0.00150 g sample of as received crude laccase was added to sterile water, stirred for an hour, and centrifuged. The supernatant was decanted off and the insoluble portion was re-suspended in sterile water. The supernatant, the re-suspended insoluble fractions, and a 0.00150 g sample of crude as received laccase enzyme were tested for enzymatic activity. This test indicated that the as received crude extract is more enzymatically

active than the fractions, with the soluble fraction exhibiting far more catalytic activity than the insoluble fraction as show in Figure 11.

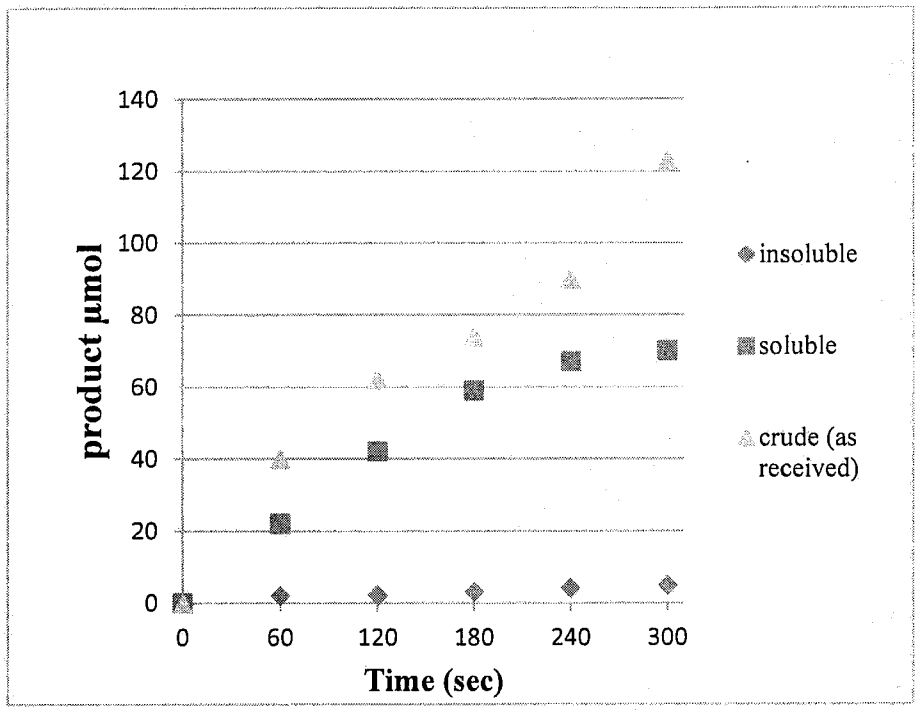


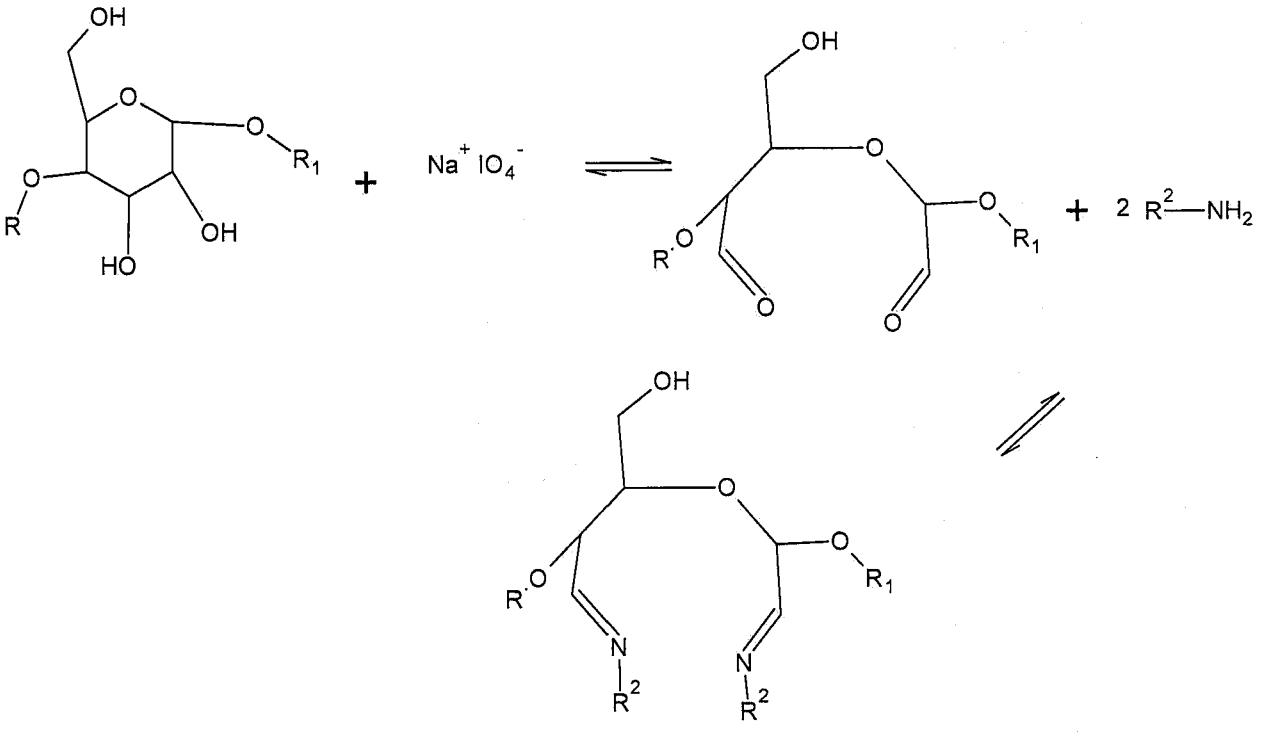
Figure 11. Catalytic oxidation of ABTS with as received laccase and the corresponding soluble and insoluble fractions versus time.

3.2 Attempted Aminopropylsilation of Bacterial Cellulose

As discussed in section 1.1 enzymes have been immobilized on support materials that have been treated with 3 aminopropyltriethoxysilane (APTES) followed by glutaraldehyde as a cross-linking agent.[10] We attempted to add amine groups to bacterial cellulose by the reaction with 3-aminopropyltriethoxysilane (APTES) in ethanol.[67] However, reactions of bacterial cellulose with APTES were unsuccessful even after varying reaction times from 5 to 10 min to 1hr, 5 h, 10 h and 24 h and the pH from 3 to 4.

3.3 Attempted Selective Oxidation of Bacterial Cellulose

As discussed in section 1.1 enzymes have been immobilized on support materials by modifying the support. We attempted to oxidize the 1,2-dihydroxyl groups located on C2 and C3 positions on the glucose ring of the amorphous sections of bacterial cellulose to dialdehydes with periodate, in a manner analogous to that used with nanocellulose as described by Varmer *et. al.*[68] The dialdehydes if formed could then be reacted with amine groups on the enzyme as shown in Figure 10. However, oxidation of the hydroxyl groups were unsuccessful even upon increasing the reaction temperature from 55 °C to 60 °C and the concentration of periodate from 0.16 M to 0.20 M. Only a minor amount of oxidation of the hydroxyl groups was observed as determine by IR. The nanocellulose used by Varmer *et. al.* was prepared by acid hydrolysis and pulp bleaching to remove the lignin and hemicellulose associated with the raw cellulose producing a nanocellulose that is lower in crystallinity and degree of polymerization than bacterial cellulose. The higher degree of polymerization and crystallinity of the bacterial cellulose may be the reason that we were unsuccessful with this partial oxidation.[69]



H_2N——R^2 R^2 = Enzyme

R and R^1 = Cellulose Chain

Figure 12. Proposed oxidation of cellulose by periodate followed by protein linkage.

3.4 Synthesis of Bacterial Cellulose in the Presence of Laccase Enzyme

In attempt to incorporate laccase enzyme into bacterial cellulose, we added laccase enzyme to the growth media for *Acetobactor xylinum* and then incubated at 27 °C. We found that adding laccase to the growth media did not interfere with the biosynthesis of cellulose. From our previous experiment as described in section 3.1, the crude as received laccase and soluble fraction are highly active for the catalytic oxidation of ABTS. Thus, we incubated *Acetobactor xylinum* in the presence of as received laccase

enzyme and the corresponding soluble fraction of the laccase enzyme. The laccase samples were added to 4 different 15.00 mL conical test tubes (duplicates of each sample) which contained growth media and *Acetobactor xylinum* cells and incubated for a total of 3 days. On the fourth day the pellicles were recovered and then washed with pH 5 phosphate buffer to remove any laccase enzyme that was not incorporated into the bacterial cellulose. The pellicles were resuspended in a solution containing ABTS and the catalytic activity was monitored as a function of time and the results are shown in Figure 13. We found that the pellicles prepared with the crude extract were far more catalytically active then the corresponding pellicle prepared from the soluble fraction. However, we could not quantitate the amount of soluble laccase enzyme utilized to prepare these pellicles. These experiments show that the both the soluble and crude laccase enzyme become incorporated in the pellicle of bacterial cellulose. The incorporation of the crude as received laccase in the cellulose can be seen in the pellicle of cellulose (Figure 14). Figure 15 shows that bacterial cellulose produced by *Acetobactor xylinum* in the absence of laccase enzyme exhibits no activity in regard to the oxidation of ABTS.

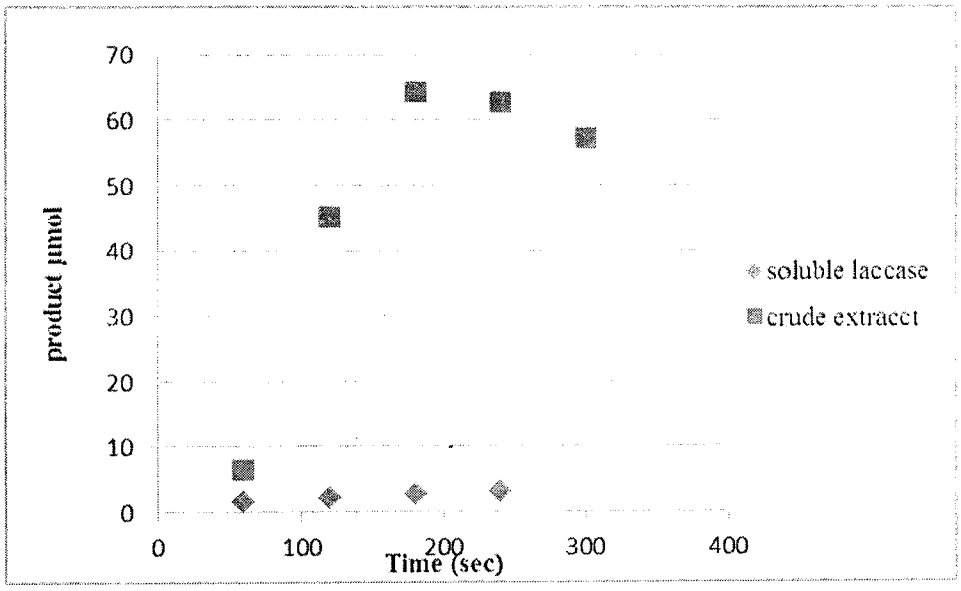


Figure 13. Catalytic oxidation of ABTS vs. time with pellicles of bacterial cellulose produced by *Acetobactor xylinum* in the presence of as received and a soluble fraction of laccase enzyme.

Figure 14. Photograph pellicles of bacterial cellulose produced by *Acetobactor xylinum* in the presence of as received laccase enzyme

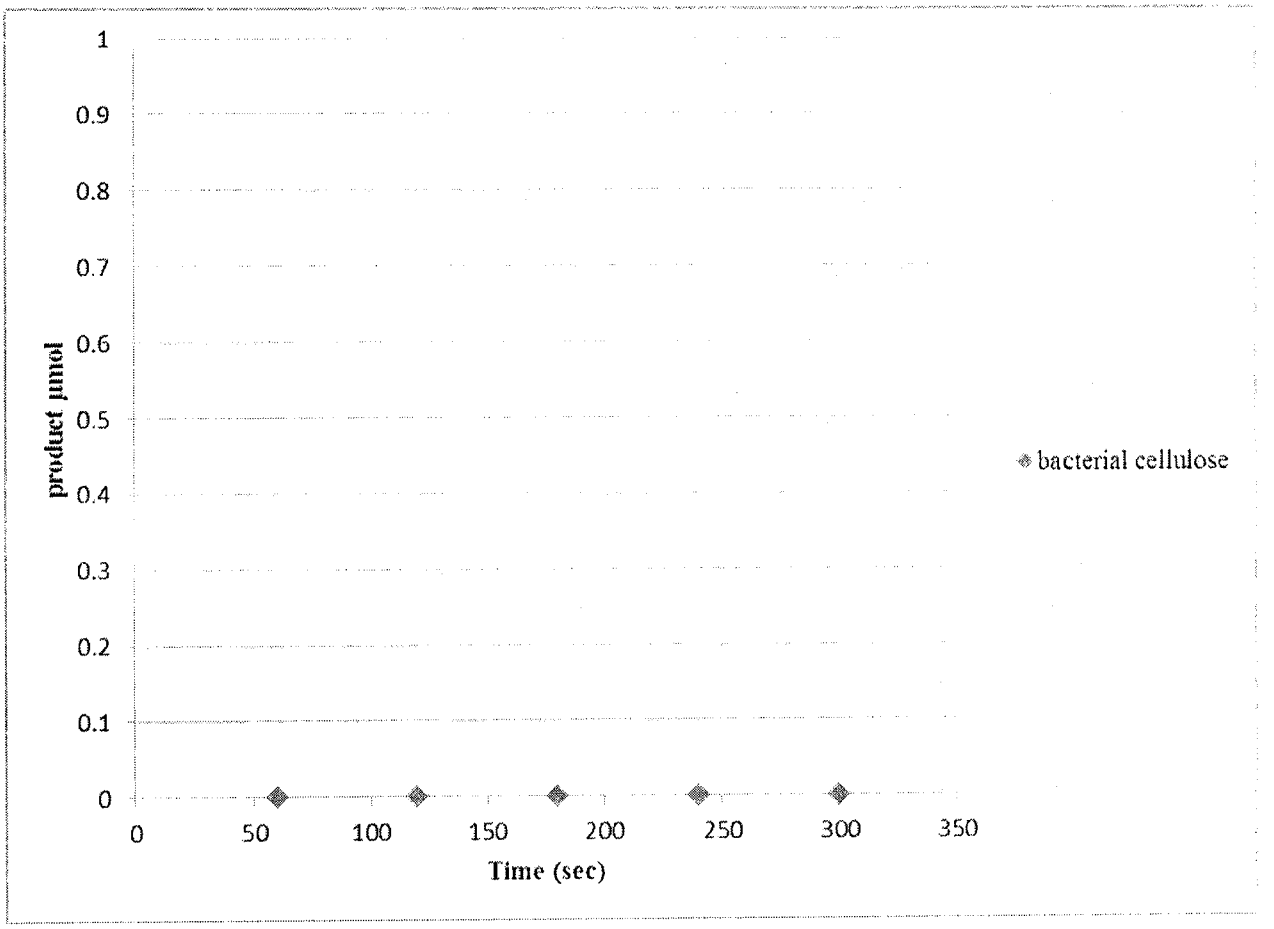


Figure 15. Catalytic oxidation of ABTS vs. time in the presence of native bacterial cellulose.

3.5 Biosynthesis of Bacterial Cellulose in the Presence of Horseradish Peroxidase

Based upon our success, as described above, in immobilizing the soluble fraction of laccase enzyme on bacterial cellulose, we decided to examine the immobilization of horseradish peroxidase, which is available as a water soluble purified enzyme, on bacterial cellulose. In a manor analogous to laccase enzyme, 1 to 10 mg of purified horseradish peroxidase was added to the growth media and *Acetobactor xylinum* was incubated to give bacterial cellulose without interfering with the production of the

bacterial cellulose. The catalytic activity of the pellicles produced in this manor are discussed in section 3.7.

3.6 Immobilization of Titanium Dioxide in Bacterial Cellulose

Based upon our success in immobilizing insoluble laccase enzyme in bacterial cellulose, as described above, we decided to examine the incorporation of an insoluble inorganic catalyst, TiO_2 in bacterial cellulose in an analogous manor. *Acetobactor xylinum* was incubated while shaken with growth media with 70μm of TiO_2 for 1, 3 and 7 days. This lead to pellicles of bacterial cellulose in which the particles of TiO_2 could be observed dispersed throughout the matrix. After two days of bacterial cellulose biosynthesis the TiO_2 was observed not to be firmly incorporated within the bacterial cellulose; however, after three days of incubation the TiO_2 was observed to be incorporated within the bacterial cellulose. It has been reported in the literature and we have also observed that when *Acetobactor xylinum* is incubated with shaking in growth media, it grows differently than when grown statically. The catalytic activity of the pellicles produced in this manor are discussed in section 3.8.

3.7 Optimization of the Immobilization of Laccase and Horseradish Peroxidase on Bacterial Cellulose.

Acetobactor xylinum was incubated in the presence of horseradish peroxidase and laccase enzyme for 1 to 36 days and the pellicles were recovered, washed and tested for catalytic activity. A set of 15 conical test tubes were filled with growth media and *Acetobactor xylinum* and incubate at 27 °C. A set of 30 conical test tubes were filled

with growth media, 0.00150 g of laccase enzyme and *Acetobactor xylinum* and incubated at 27 °C. Another set of 30 conical test tubes were filled with growth media and 0.00150 g of laccase enzyme and incubated at 27 °C. After 1, 2, 3, 4, 5, 11, 24, and 36 days one sample of the first set and two samples of sets two and three were removed from the incubator. The tubes from sets one and two had pellicles of bacterial cellulose which were recovered, washed with pH 5 phosphate buffer and tested enzymatic activity with ABTS. A 1.5 mL sample of laccase enzyme in growth media was removed from the third test tube and tested for catalytic activity with ABTS. The catalytic activity (μmol per second) of the laccase enzyme in each sample was measured by adding either the pellicle or the enzyme and growth media to a new test tube containing ABTS in pH 5phosphate citrate buffer and the reaction was followed by measuring the absorbance at 415 nm vs. time for 5 min. Figure 16 gives the catalytic activity of the various samples as a function of times in days. In all cases the bacterial cellulose grown without laccase enzyme shows no catalytic activity. The pellicles in the first two sets of tubes did not appear to increase in size or density after day 3 suggesting that the glucose has been used up or the oxygen could not permeate the bacterial cellulose phase and the bacteria has gone dormant. Figure 16 shows that the activity of the free enzyme test tube set 1 is 0.035 μmol/sec and decreasing to no activity by day 15. For test tube set two, producing the laccase immobilized on the bacterial cellulose, the activity was found to be 0.017 μmol/sec which increases to 0.028 μmol/sec on day 3 and drops to 0.015 μmol/sec on day 15, and still exhibits some enzymatic activity on day 36. The increase in enzymatic activity from day 1 to day 3 for the immobilized enzyme suggests that as more bacterial cellulose is produced more enzyme is being immobilized. The slower reduction in catalytic activity

for the immobilized vs. free laccase enzyme shows that the immobilize enzyme is more stable than the free enzyme.

Experiments with horseradish peroxidase was carried out in a manner similar to that described above for laccase; however, samples were removed from the incubator worked up tested after 1, 1.5, 2, 4, and 6 days for catalytic oxidation of phenol. In a manner similar to that above native bacterial cellulose showed no catalytic activity. The free horseradish peroxidase had an activity of 1.1 μmol/sec for the oxidation of phenol and falls slowly to 0.8 μmol/sec on day 6. The immobilized horseradish peroxidase exhibits an activity of 0.1 μmol/sec at day 1 slowly increasing at 0.2 at day 2 reaching a maximum activity of 1.18 μmol/sec on day 4, slightly higher than the free enzyme and then decreases by day 6 to 0.9 μmol/sec.

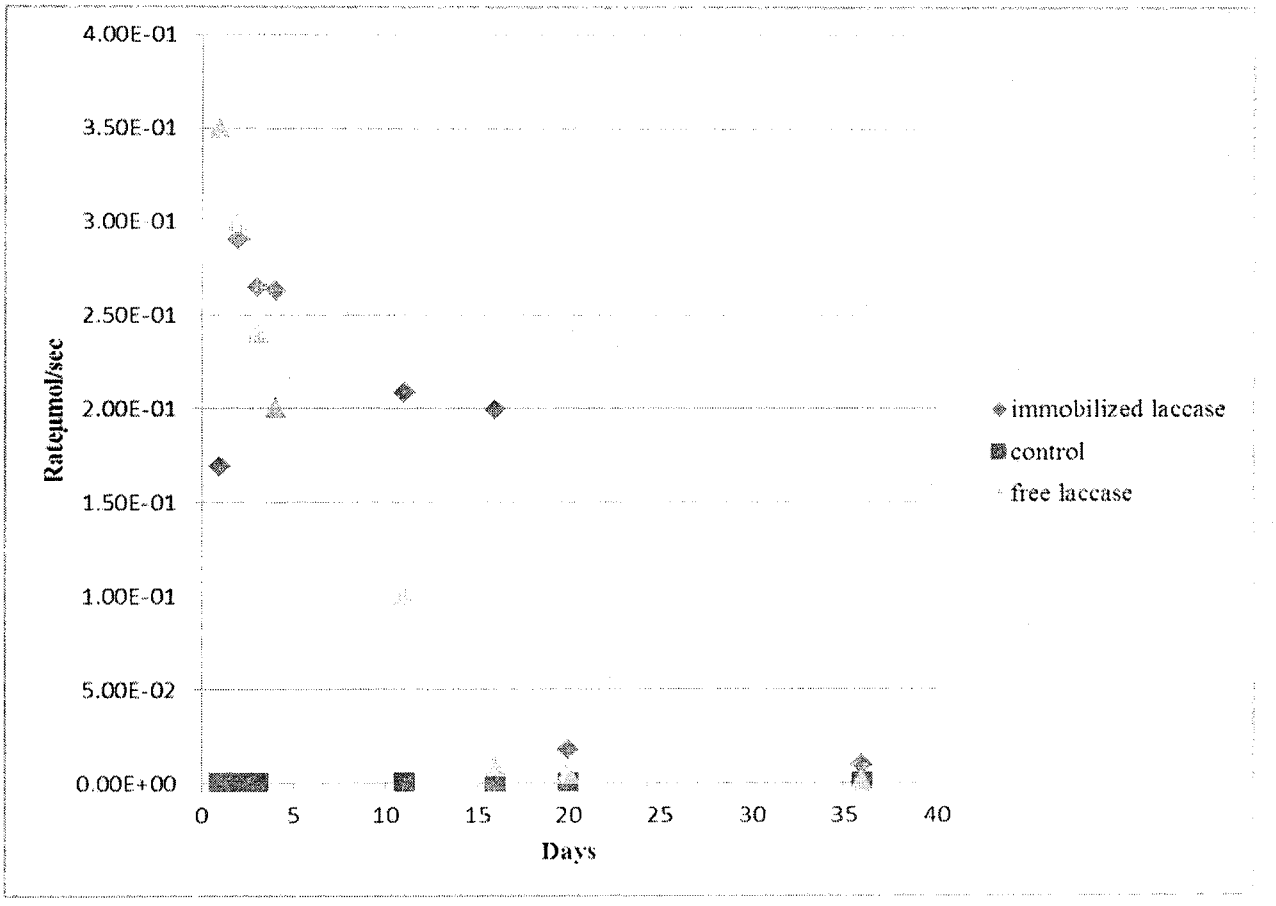


Figure 16. Catalytic activity (µmol/sec) vs. time (days) for free laccase, immobilized laccase and native bacterial cellulose.

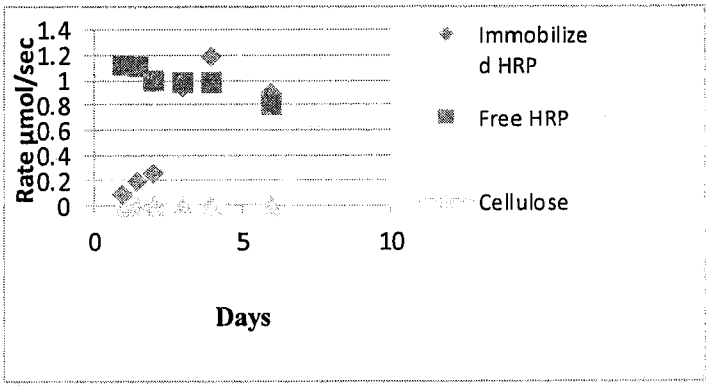


Figure 17. Catalytic activity (µmol/sec) vs. time (hours) for free horseradish peroxidase, immobilized peroxidase and native bacterial cellulose

3.8 Optimization of the Immobilization of TiO_2 on Bacterial Cellulose

Titanium dioxide is known for its photocatalytic activity which can degrade organic pollutants or inactivate microorganisms.[71] TiO_2 was incubated with *Acetobactor* xylinum in growth media for 1, 3 and 7 days as described in section 3.6. The photocatalytic inactivation of *Candida albicans* with these samples was carried as described in section 2.6. Figure 18 shows the survival (nt/n_o) of *Candida albicans*. It is observed in Figure 18 that free TiO_2 totally inactivates Candida albicans under our disinfection protocol. It also shows that TiO_2 that has been immobilized for only one day and still contains free TiO_2 also completely inactivates the *Candida albicans*. After the biosynthesis of bacterial cellulose has been carried out for 3 day the TiO_2 was fully immobilized in the bacterial cellulose and 98 % of the *Candida albicans* were inactivated during the disinfection protocol. After 7 days of biosynthesis additional cellulose had been produced further encapsulating the TiO_2 and only 82 % of the *Candida.albicans* had been photo-catalytically inactivated.

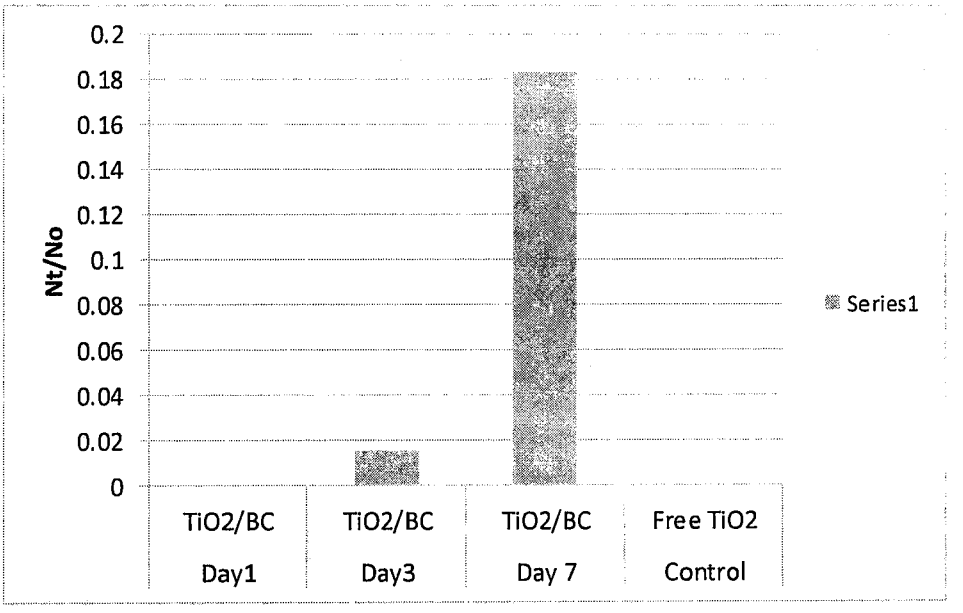


Figure 18. CFU of *Candida albicans* versus after treatment with TiO_2 immobilized with bacterial cellulose that has been grown in the presence of TiO_2 for 1, 3, and 7 days.

3.9 Reuse of Laccase and Horseradish Peroxidase Immobilized on Bacterial Cellulose

Reusing of enzymes provides cost advantages which are often an essential prerequisite for utilizing enzymes in catalytic process. Immobilization of enzymes facilitates the efficient recovery of the enzymes from reaction products, thereby minimizing or eliminating protein contamination of the product which has enabled their continuous use in a variety of reactions.[1-3]

We carried out two sets of experiments as described below to determine the potential reusability of the laccase enzyme immobilized on bacterial cellulose. In the first experiment, the oxidation of ABTS catalyzed by immobilized laccase enzyme was measured over a 5 min period and then the cellulose pellicle was removed from the

reaction mixture, washed with pH 5 phosphate buffer three times and stored in pH 5 phosphate buffer at 8 °C. This process was repeated on days 2, 4, 9, 24 by resuspending the sample of laccase immobilized on cellulose in an ABTS solution and measuring the activity (μmol/sec) Figure 19 shows that the activity dropped dramatically within the first 3 days, and more slowly over the next 20 days. In the second experiment, the oxidation of ABTS catalyzed by immobilized laccase enzyme was measured over a 5 min period and then the cellulose pellicle was removed from the reaction mixture, washed with pH 5 phosphate buffer three times and the enzymatic assay was immediately repeated. This process was repeated for 11 cycles. Figure 20 shows the catalytic activity of the immobilized laccase as a function of cycles. Over the first five cycles, the enzymatic activity rapidly decreases from 0.3 to 0.06 μmol/sec and then is fairly stable over the next 6 cycles. The combination of these two experiments suggests that the number of times the enzyme is used is more important than the storage time when determining the catalytic activity.

We carried out a similar set of experiments for horseradish peroxidase immobilized on bacterial cellulose. Figure 21 shows that the activity of the horseradish peroxidase drops dramatically during the first cycle of use and storage and similarly shows a dramatic drop in activity upon cycling without storage.

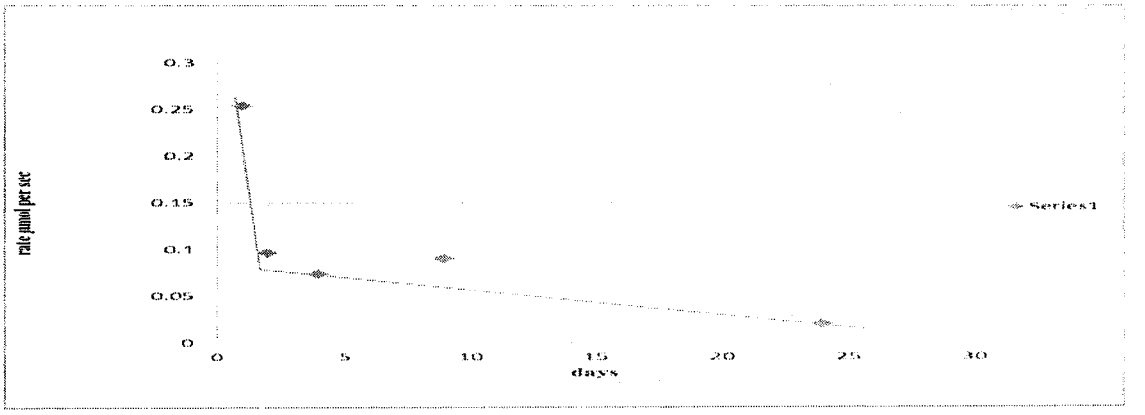


Figure 19. Catalytic activity (µmol/sec) of laccase immobilized on bacterial cellulose for the oxidation of ABTS vs. use and days of storage (days).

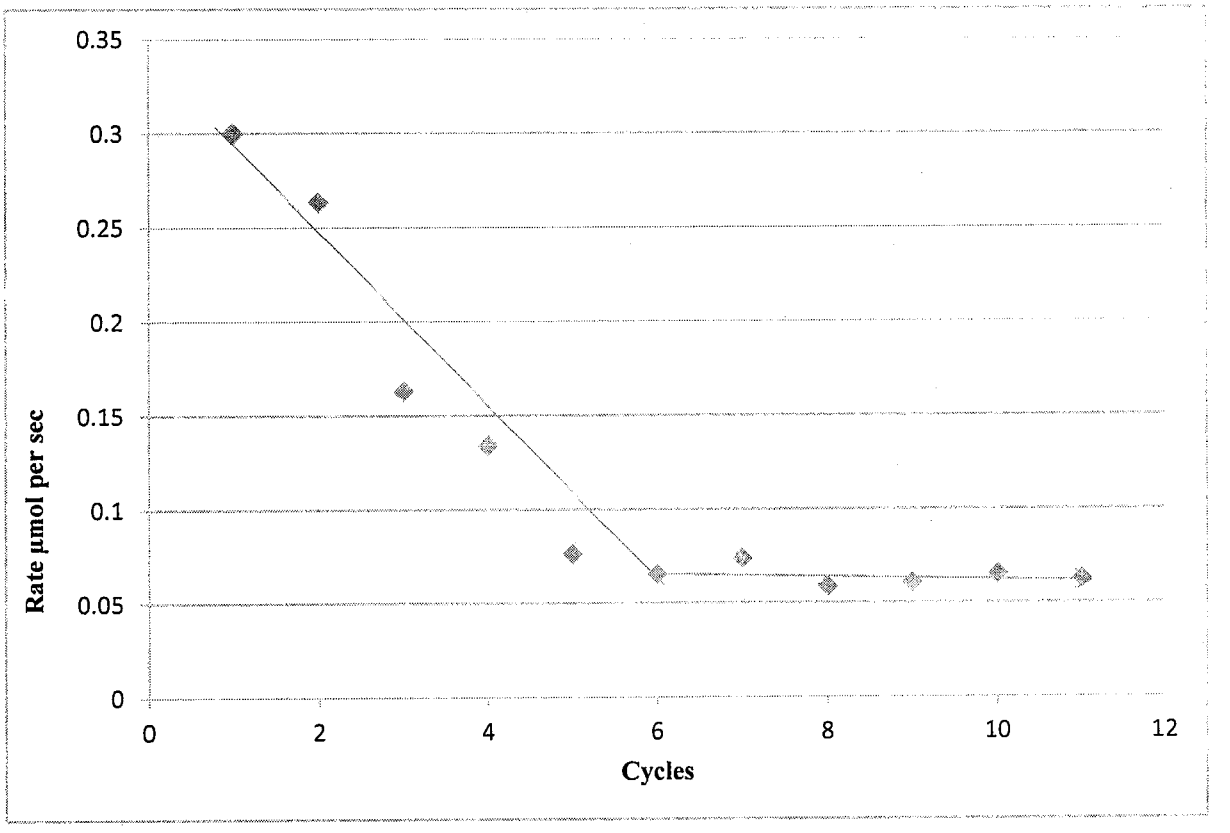


Figure 20. Catalytic activity (µmol/sec) of laccase immobilized on bacterial cellulose for the oxidation of ABTS vs. use.

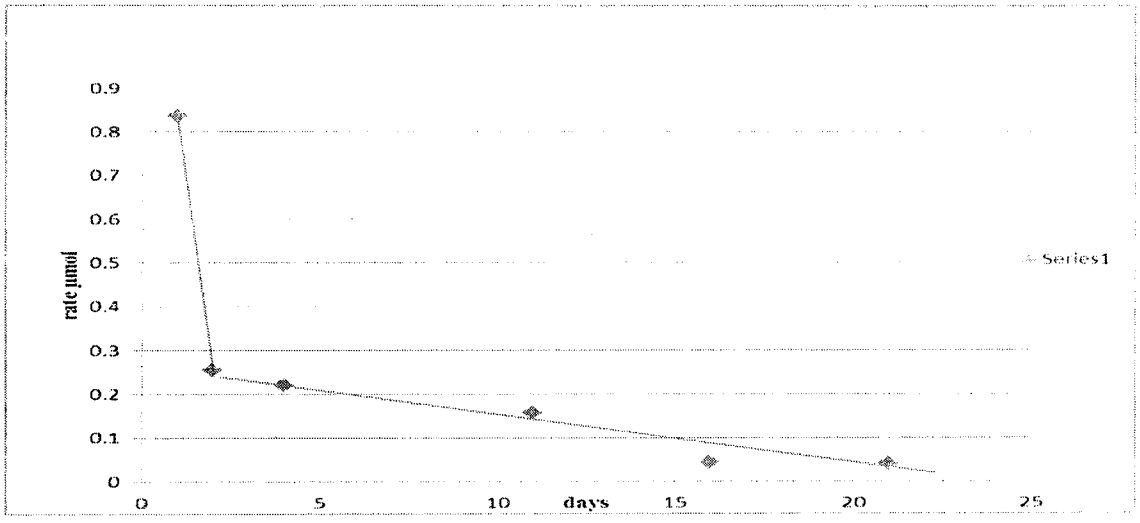


Figure 21. Catalytic activity (µmol/sec) of horseradish peroxidase immobilized on bacterial cellulose for the oxidation of phenol vs. use and storage.

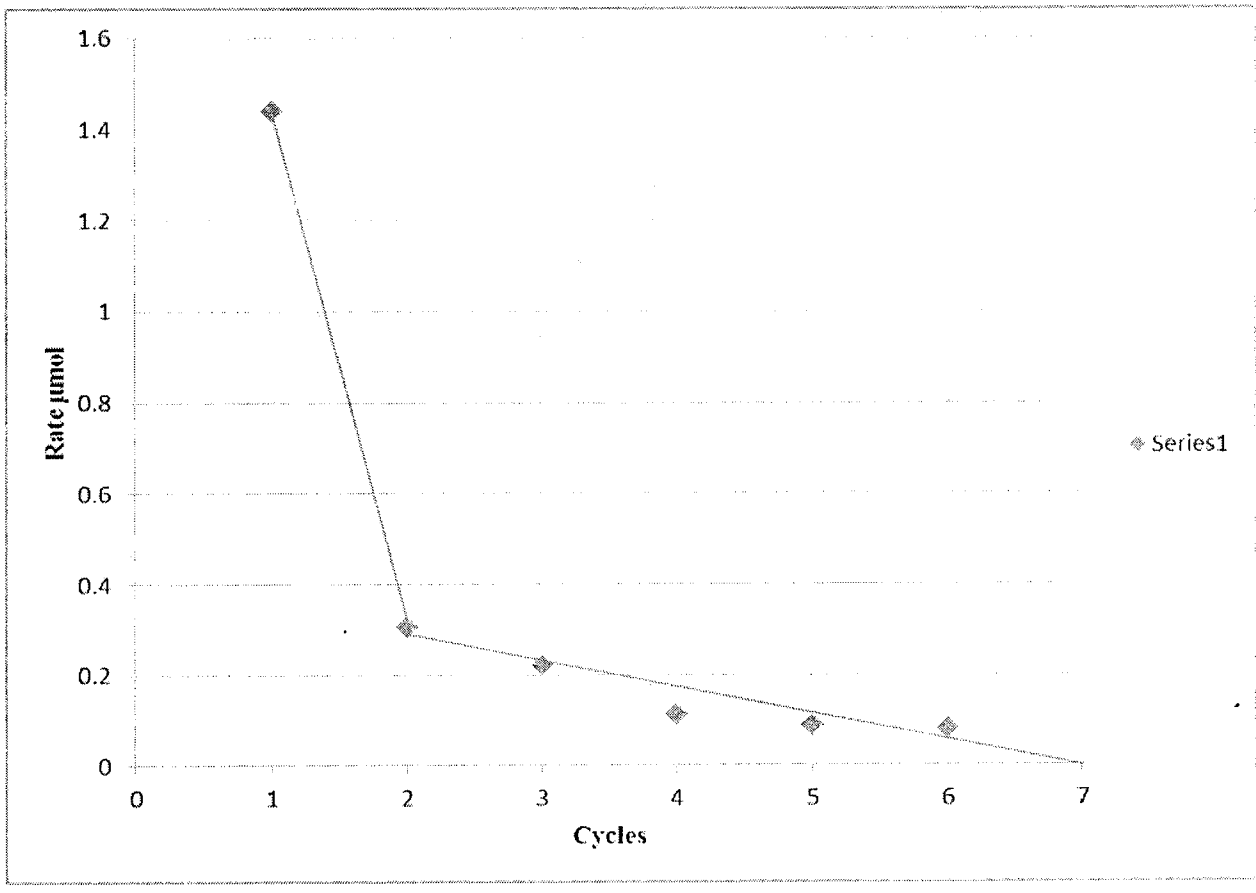


Figure 22. Catalytic activity (µmol/sec) of horseradish peroxidase immobilized on bacterial cellulose for the oxidation of phenol vs. use.

Laccase enzyme has been immobilized on many different support materials in an attempt to increase stability and reusability.[17,39,40] Mogharabi *et al.* immobilized laccase by entrapment in an alginate gelatin mixed gel and the immobilized laccase retained more than 85% activity after five successive cycles of use.[39] Lu *et al.* immobilized laccase on alginate-chitosan microcapsules and the immobilized laccase retained more than 35 % of its initial activity after three batch uses.[39] In our immobilized system, laccase retained more than 50 % of its initial activity after 11 consecutive cycles, showing this method aids in retaining activity of the enzyme after multiple uses. Mohammed *et al.* immobilized horseradish peroxidase on a composite membrane by coating it with nonwoven polyester fabric and chitosan glutamate then cross linked the enzyme to this carrier using gluteraldehyde.[48] They were able to reuse the immobilized horseradish peroxidase for 10 cycles and retained 54 % of its activity. Typically horseradish peroxidase is known to lose the majority of its activity after one use.[72] We found that horseradish peroxidase immobilized on bacterial cellulose lost 76 % of its activity after one cycle of use, however it exhibited activity after 5 cycles of use.

3.10 Oxidation of 2 Chlorophenol by Immobilized TiO_2 and Horseradish Peroxidase

Chlorophenols are organochlorides that contain one or more covalently bonded chlorine atoms.[59] Chlorophenols are toxic compounds that are present in drinking-water and the removal of these compounds by free and immobilized horseradish peroxidase along with Titanium dioxide has been extensively studied. The mechanism by which these two catalysts remove these phenolic compounds in water differs. The horseradish –

catalyzes a one electron oxidation of phenolic compounds and generates free aromatic radicals which combine to form polymers of low solubility that eventually precipitate from solution.[8] The horseradish peroxidase-mediated oxidative coupling process has the potential for remediation of aqueous solution contaminated by phenolic compounds; however, this process is hampered by the low operational stability of horseradish peroxidase.[7] This is considered a major drawback of the current enzymatic approach. To enhance the efficacy and cost effectiveness of the enzymatic approach, various additives have been tested for their ability to minimize enzyme inactivation. These studies have shown that the oxidation of phenols catalyzed by peroxidases can be enhanced by adding coagulants.[73,74,75] To avoid polymerization by horseradish peroxidase, the Emerson reaction was implemented by adding 4-aminoantipyrine to the reaction mixture. This reaction is considered the most reliable for colorimetric evaluation of phenolic compounds after they have undergone a one electron oxidation.[75] Figure 23 shows the percentage of chlorophenols removed from solution vs. time upon treatment with bacterial cellulose, horseradish peroxidase immobilized on bacterial cellulose and free horseradish peroxidase. Over a 10 min period bacterial cellulose absorbed less than 5% of chlorophenol in solution. The immobilized horseradish peroxidase removed 76% of the chlorophenol while free horseradish peroxidase removed 84% of the chlorophenol (Figure 23). Tatsumi *et al.*, used horseradish peroxidase immobilized on magnetite to remove chlorophenols from waste water. This system removed almost 100% of chlorophenol; however, they discussed that magnetite that the horseradish peroxidase was immobilized on contributed significantly to the chlorophenol removal.[50]

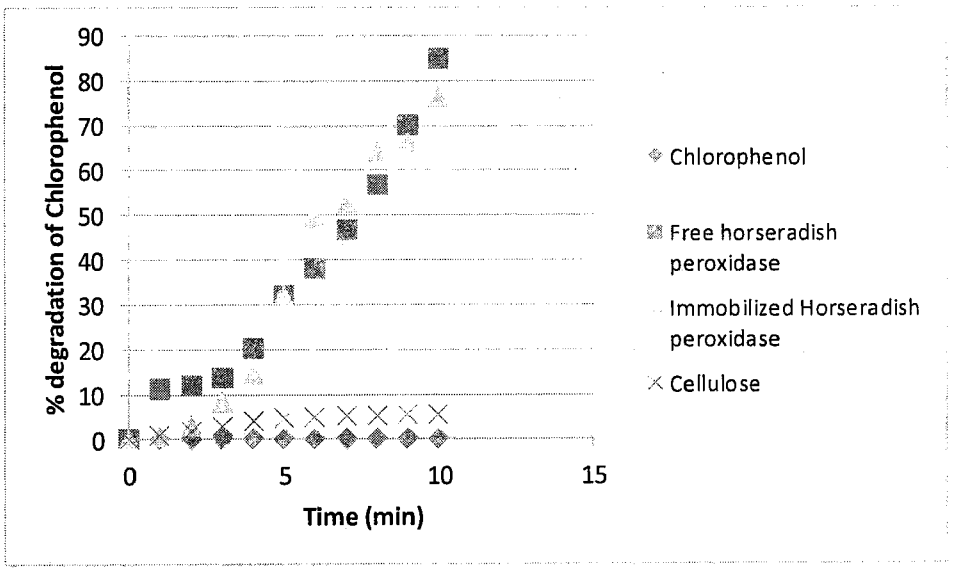


Figure 23. Removal of 2-Chlorophenol vs. time with bacterical cellulose, horseradash peroxidiase and immobilided horseradish peroxidase.

The photocatalyst titanium dioxide has also been used to remove chlorophenols from waste water. Most investigators have suggested that photocatalytic degradation of chlorinated phenols occurs by reductive decomposition by electrons transferred from the conduction band since the release of chloride ion was detected, whereas no polymerization product was identified.[76] Thus we used a the UV absorbance of the chlorophenol to follow the reaction rather than a colorimetric assay. In this study, TiO_2 immobilized on cellulose removed about 65% of chlorophenol compared to the free TiO_2 which removed 100% of the chlorophenol as show in Figure 24. Even though the immobilized TiO_2 was less active than the free TiO_2, the removal of the free TiO_2 particles from water was difficult where as the immobilized TiO_2 was readily recovered.

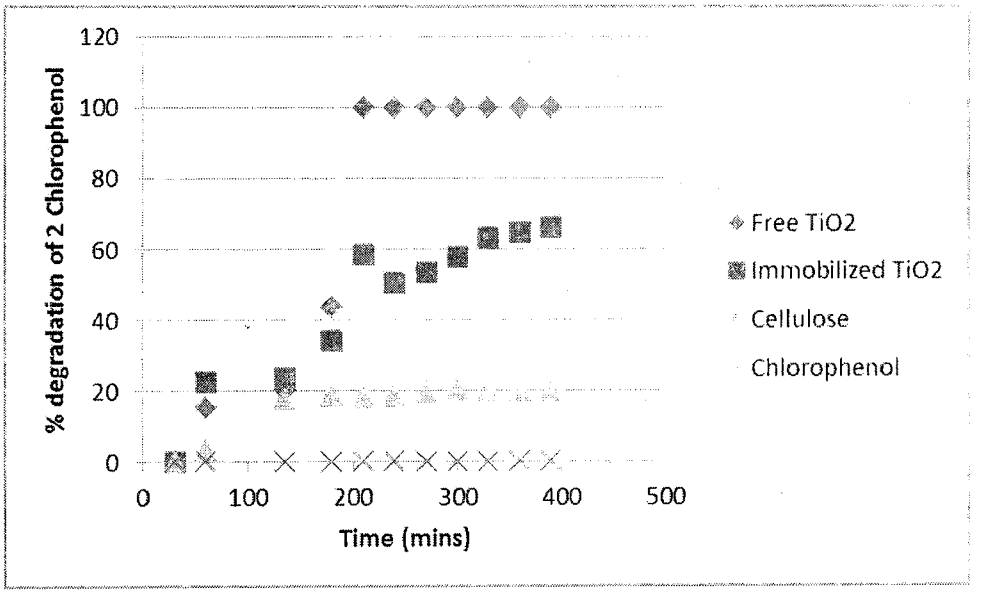


Figure 24. Removal of 2-Chlorophenol vs. time with bacterical cellulose, TiO_2 and immobilided TiO_2 under photolysis.

3. 11 Characterization of the Interaction Between the Horseradish Peroxidase and Bacterial Cellulose

Carpenter *et al.* used infrared spectroscopy to determine the interaction of proteins with sugars. IR provides a convenient and a powerful means to monitor hydrogen bonding between two molecular species in the solid state. In their study they discuss how alterations in the vibrational spectra of carbohydrates dried in the presence of proteins and the complementary influence of carbohydrates on dried protein spectra are indicative of sugar-protein hydrogen bonding.[77] When determining hydrogen bonding by carbohydrates, the feature of the infrared spectrum most often monitored is the hydroxyl

stretching region, found from 3650 to 3100 cm^{-1}. The immobilized horseradish peroxidase on cellulose may follow this trend because this enzyme is an N-linked glycoprotein and could potentially have a sugar sugar interaction between mannose from the horseradish peroxidase and glucose in the cellulose. The Alcohol on carbon 6 on the mannose ring and the hydrogen of off carbon 3 on the glucose ring could be hydrogen bonded to attach the horseradish peroxidase to the cellulose. This bond does not interfere with the active site or R-groups associated with the amino acids present on the enzyme (figure 25). The FT-IR spectrum below shows the immobilized horseradish peroxidase on bacterial cellulose, free horseradish peroxidase and bacterial cellulose (figure 26). The peak broadens between 3270 cm^{-1} and 3344 cm^{-1} on the immobilized horseradish peroxidase spectra which could indicate that there is intermolecular hydrogen bonding between the carbohydrate molecules. This is not seen in the bacterial cellulose, however for the free horseradish peroxidase there is a peak at 3263 cm^{-1} that could be due to the hydrogen bonding within the molecule. The large widths of these bands are thought to arise from coupled vibrations rather than from the separate vibrations of individual groups. The position of the broad band is indicative of the type of hydrogen bonding in which the constituent hydroxyl groups are involved.[77] For example, Carpenter *et al.*, showed the OH stretching bands for dried trehalose, lactose, and myo-inositol are centered around 3350 cm^{-1}. In the immobilized horseradish peroxidase there is a peak at 3345 cm^{-1}, which could be the OH stretching on the mannose sugar on the horseradish peroxidase and the OH on the cellulose. This indicates intermolecular hydrogen bonding between the carbohydrate molecules.This is not seen in any of the other peaks for

bacterial cellulose and free horseradish peroxidase which could be because there is no intermolecular hydrogen bonding associated with these molecules.

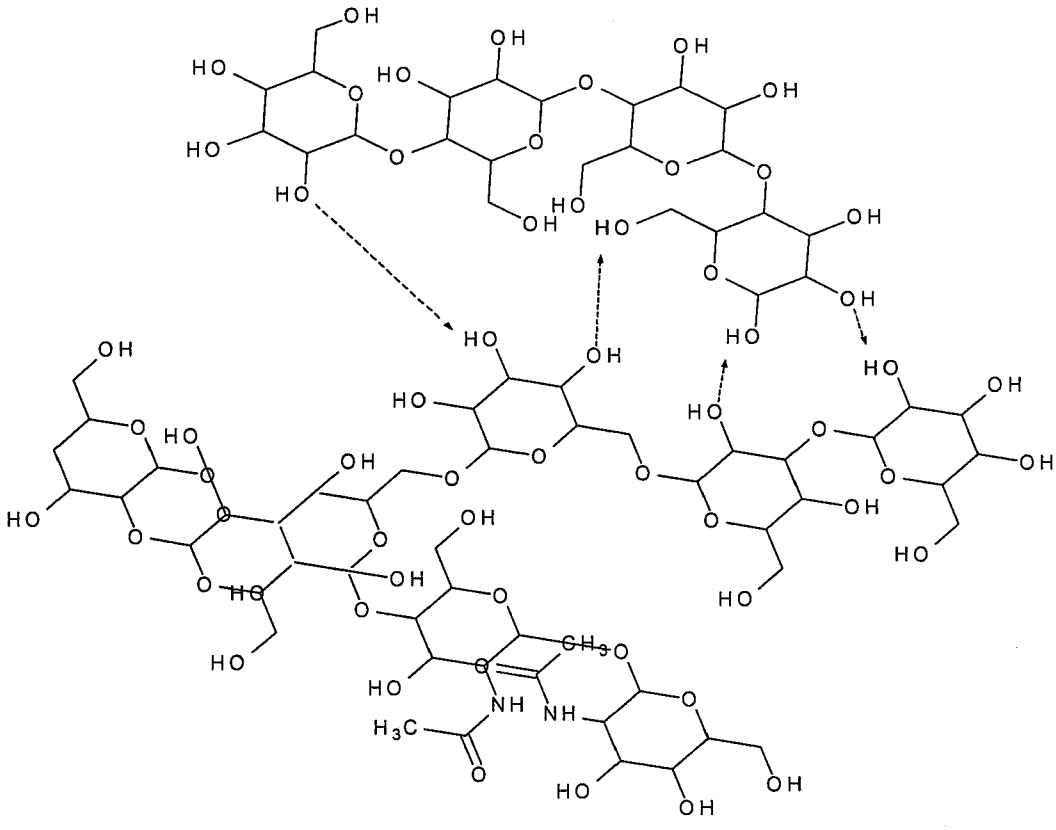


Figure 25. Penta-saccharide core of all N-linked glycoproteins hydrogen bonded to bacterial cellulose.

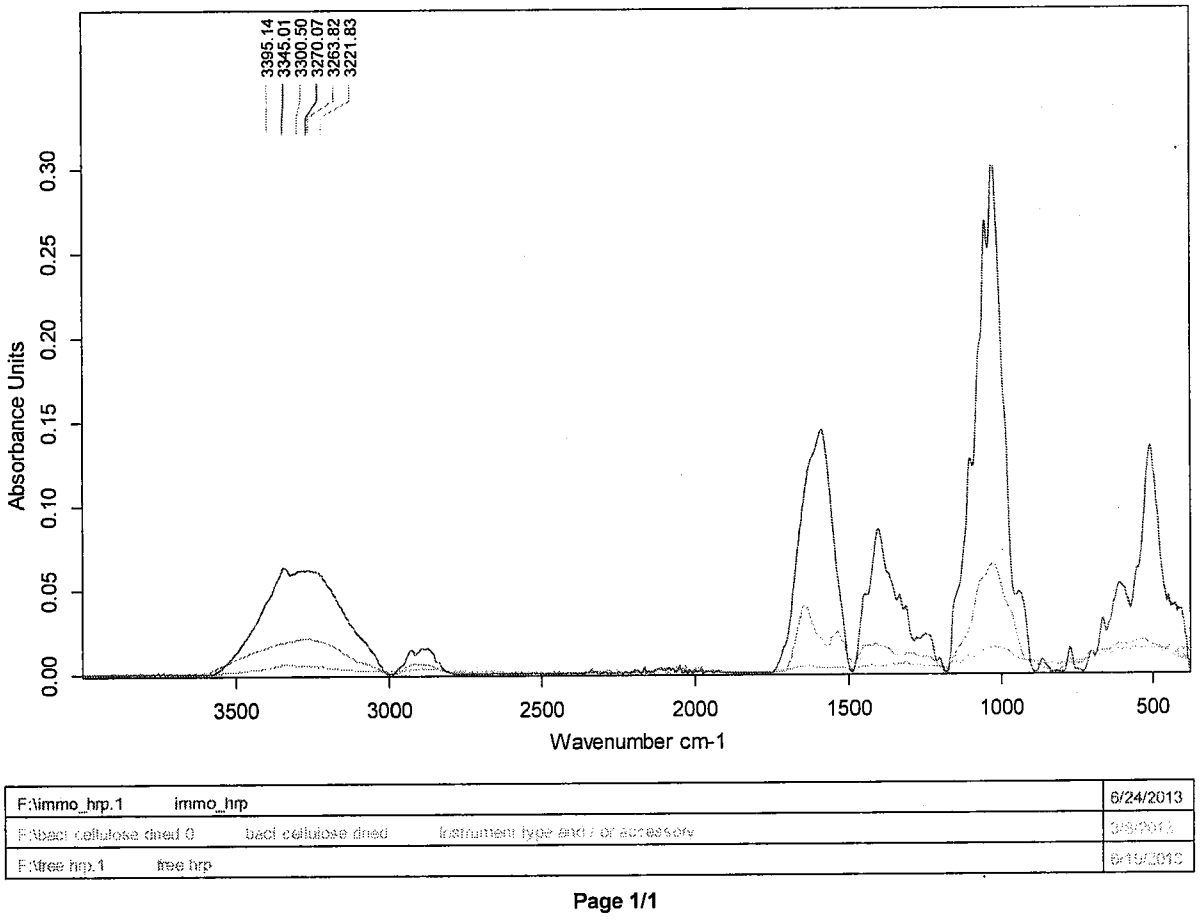


Figure 26. FT-IR of free horseradish peroxidase compared to immobilized horseradish peroxidase.

CHAPTER 4

CONCLUSION

4.0 Conclusion

Immobilization of catalyst by synthesizing cellulose produced by *Acetobactor xylinum* in the presence of laccase, horseradish peroxidase and photocatalyst titanium dioxide was successfully carried out. This new method increased stability and functionality of the catalyst compared to the free catalyst and allowed the catalyst to be reused. Not only were these catalyst immobilized, they were catalytically active and were able to be used in the removal of chlorophenols. The retained activity of the immobilized enzymes was comparable to what is reported in the literature and could be used as a potential method to immobilize a variety of catalyst on bacterial cellulose.

4.1 Recommendations for future work

Future work includes increasing the concentration of the carbon source in the media for the *Acetobactor xylinum*. This could allow more catalyst to get immobilized on the bacterial cellulose, if more cellulose can be synthesized. If successful, this could potentially expand the type of substrate being incorporated in the cellulose.

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